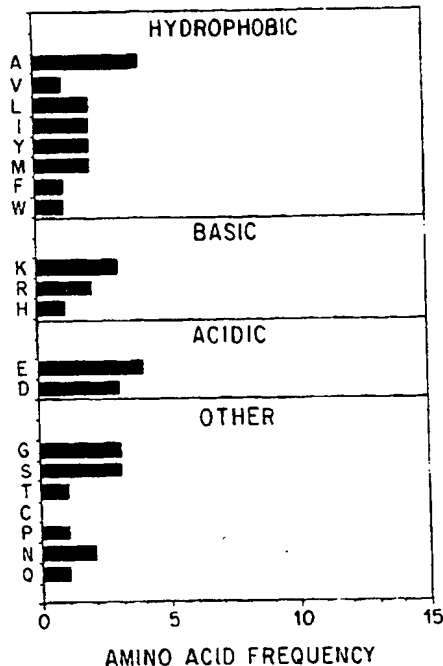
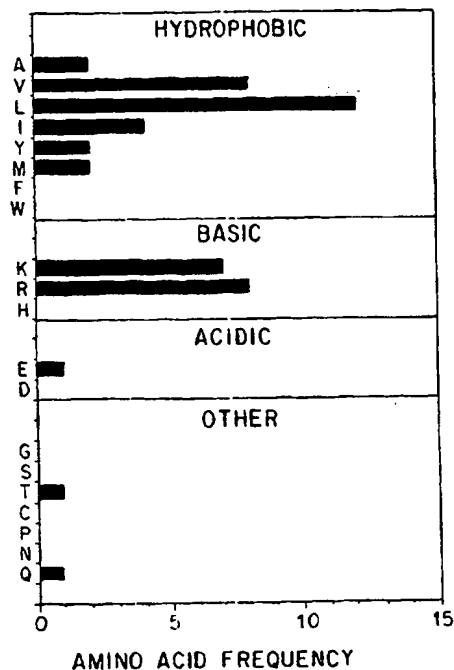




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(57) Abstract

Described herein are methods and drugs that inhibit the processing of antigens for presentation by major histocompatibility complex class I molecules. Specifically, inhibitors of the ATP-ubiquitin dependent proteolytic pathway are described, which can inhibit MHC-I antigen presentation. These methods and drugs may be useful for the treatment of autoimmune diseases and for reducing rejection of organs and graft transplants.

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-1-

ROLE OF ATP-UBIQUITIN-DEPENDENT PROTEOLYSIS IN  
MHC-1 RESTRICTED ANTIGEN PRESENTATION  
AND INHIBITORS THEREOF

DESCRIPTION

5 Background

There are many diseases that result from the immune system attacking and destroying tissues of the body. These conditions can cause severe morbidity and in many cases can be fatal. Some of these diseases, such as  
10 systemic lupus erythematosus, result from an individual's immune system reacting against its own cells as if they were foreign or pathogenic. Collectively these diseases are referred to as autoimmune diseases. Other diseases result from the  
15 immune system responding against molecules, such as therapeutic drugs, that are present in the body's tissues. Yet other diseases are caused when the immune system responds against transplanted foreign tissues in the body. The immune response against foreign tissues  
20 is the major cause of failure of transplanted organs or grafts in transplantation and is the principle barrier to this procedure.

In these diseases, the cells that cause the destructive immune response are lymphocytes, and in the  
25 vast majority of cases, T lymphocytes play an obligatory role in these responses. The immune response is initiated when T lymphocytes recognize fragments of antigens that are bound to major histocompatibility complex (MHC) molecules on the surface of antigen-  
30 presenting cells. The antigenic peptide bound to the MHC molecules stimulates the T cells and directs them to

-2-

respond against the cells bearing the antigen-MHC complex.

There are several different kinds of T lymphocytes. The two major subsets of T lymphocytes, which are distinguished by the expression of certain cell surface molecules, differ in their specificity and function. T cells that express the CD4 molecule are specific for antigens presented by MHC class II molecules and usually stimulate antibody or inflammatory responses. T cells that express CD8 molecules are specific for antigens presented by MHC class I molecules and function to kill host cells. The CD8<sup>+</sup> T cells mediate tissue damage in several of the immune mediated diseases described above.

The therapeutic approaches to treating the immune-mediated diseases are directed at blocking the response of the lymphocytes. Some therapies, such as anti-lymphocyte globulin, seek to eliminate T cells. Other therapies, such as cyclosporin A and steroids, seek to block the response of lymphocytes by inhibiting lymphocyte activation. Still other therapies, such as treatment with monoclonal antibodies against cell interaction molecules, seek to block lymphocytes from interacting with other cells. All of these therapies have significant adverse side effects. Several of the drugs have associated toxicities; e.g., cyclosporin is associated with nephrotoxicity, and steroids cause Cushing's disease. Monoclonal antibodies are associated with immune responses against the antibodies, which makes continued therapy ineffective. In all cases, immunosuppression results in increased susceptibility to infection, which can be lethal. A better approach to controlling or altering T cell responses would be very beneficial therapeutically.

-3-

Summary of the Invention

Described herein are methods and drugs for blocking cytolytic immune responses, which are useful for the treatment of a variety of autoimmune diseases and for preventing rejection of transplanted organs or grafts. These methods and drugs block the generation of cellular immunity by preventing the initial presentation of cellular and viral antigens on MHC-class I molecules to T cells. The methods comprise using inhibitors of the ATP-ubiquitin-dependent proteolytic pathway to prevent or reduce the processing of intracellular proteins into the antigenic peptides that bind to MHC-1 molecules.

This invention results from Applicants' work, which shows that inhibition of an early step (ubiquitin activation) and a late step (cleavage of intracellular proteins into peptides) in the ATP-ubiquitin-dependent proteolytic pathway can inhibit MHC-1 antigen presentation by blocking the generation of peptides binding to MHC-1 molecules. This work provides strong evidence for the role of this cytosolic protein degradation system in the processing of MHC-1 presented antigens for MHC-1 presentation. Furthermore, Applicants have discovered new features of the function of the proteasome (multicatalytic protease complex), an essential component of the ATP-ubiquitin pathway. As a result of their work, methods and drugs are provided for reducing cytolytic immune responses without affecting antibody-mediated or inflammatory responses. This invention should thus provide therapeutics useful for autoimmune diseases and prevention of transplant rejection without the attendant disadvantages of generalized immune suppression.

-4-

Specifically, Applicants have discovered that gamma interferon ( $\gamma$ -IFN), which has long been known to stimulate antigen presentation, alters the relative activities of the multiple peptidases of the proteasome, so as to promote the generation of peptides with the characteristic carboxyl-terminal residues found on MHC-1 bound peptides. The trypsin-like peptidase, which cleaves on the carboxyl side of basic residues, and the chymotrypsin-like peptidase, which cleaves after hydrophobic residues, were found to have increased activity, while the peptidylglutamyl peptidase, which cleaves after acidic residues, had decreased activity. Furthermore this effect of  $\gamma$ -IFN on the proteasomes is dependent on the expression of two genes at the MHC locus, LMP 2 and LMP 7.

Secondly, Applicants have demonstrated that MHC-I antigen presentation can be blocked by inactivation of ubiquitin conjugation, an early step in the ATP-ubiquitin-dependent proteolytic pathway. It was found that, at the nonpermissive temperature, class I-restricted antigen presentation was inhibited in a mutant cell line expressing a thermolabile ubiquitin activating enzyme (E1).

Applicants have further demonstrated that chymostatin, an inhibitor of chymotrypsin-like proteases, inhibits MHC-I presentation by antigen presenting cells. Other inhibitors, particularly of the chymotrypsin-like and trypsin-like activities of proteasomes, are further described herein.

#### Brief Description of Drawings

Figure 1 shows the effect of gamma interferon ( $\gamma$ -IFN) on peptidase activities of proteasomes from U937

-5-

cells. The upper panel shows the hydrolysis of the indicated substrates by proteasome fractions from control cells and cells treated with  $\gamma$ -IFN. The lower panel shows kinetic analysis of rates of cleavage of the substrates by the same proteasome fractions.

Figure 2 shows the effect of  $\gamma$ -IFN on peptidase activities of purified 20S and 26S proteasomes.

Figure 3 shows the difference in activities of the peptidases of proteasomes from wild-type and MHC-deficient lymphoblastoid cells. The upper panel shows the peptidase activities. The lower panel shows kinetic analysis.

Figure 4 shows the frequency of amino acid residues preceding bonds cleaved to generate peptides presented on MHC-class I molecules. The left panel shows the frequency of amino acids at the carboxyl-termini. The right panel shows the frequency of amino acids preceding the amino-terminal residues.

Figure 5 (A-D) shows MHC class I-restricted presentation of ovalbumin (OVA) by mutant (ts20.10.2) and wild-type (E36.12.4) cells.

Figure 6 (A-B) shows the effect of temperature on the synthesis and maturation of H-2K<sup>b</sup> MHC heavy chains.

Figure 7 (A-B) shows MHC class I-restricted presentation of endogenously synthesized OVA<sub>257-264</sub> peptide.

Figure 8 shows inhibition of MHC class I-restricted presentation of ovalbumin (left) and ovalbumin peptide (right) by chymostatin.

### 30 Detailed Description

This invention relates to an approach for inhibiting cytolytic immune responses that avoids

-6-

generalized suppression of T lymphocyte function with its attendant risks of infection. The methods and drugs of this approach are useful for treating autoimmune diseases and preventing rejection of foreign tissues, such as transplanted organs or grafts. The strategy is to inhibit antigen presentation by major histocompatibility complex (MHC) I molecules rather than suppress T cell activity. This approach has the advantage that it selectively affects only class I MHC-restricted immune responses and not antibody or other CD4<sup>+</sup> T cell-mediated responses. Consequently, there should be less generalized immunosuppression and susceptibility to infection in the patient. Drugs that interfere with class I antigen presentation should also be useful in combination with other existing therapies and may lower the dose used in these therapies, which would decrease toxicities.

Specifically, methods and drugs are described that inhibit the processing of internalized cellular or viral antigens into the kind of peptides, referred to as antigenic peptides, that bind to the MHC-I molecules. MHC-I binding peptides have strict sequence and size requirements. In the absence of the antigenic peptides, the antigens are not presented at the cell surface and CD8<sup>+</sup> T cells are not stimulated. This method should block both the initiation of immune response and stop ongoing immune responses.

This invention is based on Applicants' work (see Examples below) supporting the role of the ATP-ubiquitin dependent proteolytic pathway, and in particular, proteasomes (multicatalytic proteinase complexes), in the generation of MHC-I-associated antigenic peptides. An initial step in the presentation of intracellular and



-7-

viral proteins to the immune system is their proteolytic degradation in the cytosol to small peptides (Townsend and Bodmer, 1989). The antigenic fragments are then taken up via specific membrane transporters (Monaco, 5 1992; Powis, et al., 1991; Spies and DeMars, 1991) into the endoplasmic reticulum, where they associate with the MHC-I molecules. These peptide-protein complexes are then transported to the cell surface for presentation to cytotoxic T cells. The cleavage of proteins into 10 antigenic peptides seems to be a regulated process, since there are strict size and sequence constraints on the peptides that can bind to MHC-class I molecules. The identity of the cleavage enzymes and the nature and precise location(s) of the proteolytic steps that 15 generate the 8-9 residue peptides presented on MHC-class I molecules have not been definitively established, although it is clear that the proteolysis does not occur in lysosomal or endosomal compartments (Morrison et al., 1986).

20 Recent studies have implicated the proteasomes, an essential component of the ATP-ubiquitin-dependent proteolytic pathway, in the generation of antigenic peptides. Several proteolytic systems exist in eukaryotic cells, and the ATP-ubiquitin pathway is the 25 major cytosolic pathway of protein degradation (Finley and Chau, 1991; Hershko and Ciechanover, 1982; Rechsteiner, 1987). In this multistep process, protein substrates are first modified by covalent conjugation to multiple ubiquitin moieties, which marks them for rapid 30 degradation by a 26S (1,500 kD) ATP-dependent proteolytic complex, called the 26S proteasome or UCDEN (Goldberg and Rock, 1992; Goldberg, 1992; Tanaka et al., 1992; Waxman et al., 1987; Hough et al., 1987). This

-8-

large structure contains the 20S (about 700 kD) proteasome as its proteolytic core plus many additional regulatory and catalytic components (Goldberg and Rock, 1992; Orlowski, 1990; Rivett, 1989; Waxman et al., 1987; Hough et al., 1987; Armon et al., 1990; Driscoll and Goldberg, 1990; Eytan et al., 1989). The precise intracellular function of the 20S proteasome is not clear; when isolated, this particle can degrade proteins and oligopeptides, but not ubiquitin-conjugated proteins (Goldberg, 1992; Tanaka et al., 1992; Armon, 1990; Driscoll and Goldberg, 1990a; Eytan et al., 1989; Driscoll and Goldberg, 1989).

The 20S proteasome is composed of about 15 distinct subunits of 20 - 30 kD. It contains three or four different neutral peptidases, which cleave specifically on the carboxyl side of hydrophobic, basic and acidic amino acids (Goldberg and Rock, 1992; Goldberg, 1992; Tanaka et al., 1992; Orlowski, 1990; Rivett, 1989). These peptidases are referred to as the chymotrypsin-like peptidase, the trypsin-like peptidase, and the peptidylglutamyl peptidase, respectively. Which subunits are responsible for these activities is unknown, although the cDNAs encoding several subunits have been cloned (Tanaka et al., 1992).

Recent studies have found that the 20S proteasomes resemble in size and subunit composition the MHC-linked low molecular weight protein (LMP) particles (Driscoll and Finley, 1992; Goldberg and Rock, 1992; Monaco and McDevitt, 1986; Parham, 1990; Martinez and Monaco, 1991; Oritz-Navarette et al., 1991; Glynne et al., 1991; Kelly et al., 1991; Monaco and McDevitt, 1982; Brown et al., 1991; Goldberg, 1992; Tanaka et al., 1992). The LMP particles contain two polypeptides, LMP 2 and LMP 7,

-9-

which are encoded in the MHC chromosomal region. Treatment of cells with gamma interferon ( $\gamma$ -IFN) stimulates antigen presentation (Townsend and Bodmer, 1989; Yewdell and Bennink, 1992) and causes the

5 induction of LMP 2 and LMP 7, as well as other MHC genes (Glynne et al., 1991; Kelly et al., 1991; Monaco and McDevitt, 1982; Yang et al., 1992). Immunochemical studies strongly suggest that LMP 2 and LMP 7 are two

10 subunits of particles representing a small fraction of the 20S proteasome population. However, the importance of these subunits in the immune response is uncertain, since deletion of these genes does not prevent antigen presentation, in contrast to deletion of other MHC-genes

15 (Arnold et al., 1992; Mamburg et al., 1992). Moreover,  $\gamma$ -IFN can alter the polypeptide composition of 20S and 26S proteasomes (Yang et al., 1992) in mutants lacking the LMP 2 and 7. It has, thus, been a matter of uncertainty whether proteasomes play a role in antigen presentation (see discussions in Goldberg and Rock,

20 1992; Driscoll and Finley, 1992; Yewdell and Bennink, 1992, pp. 26-28).

Applicants' work described herein provides confirming evidence for a role for the 20S and 26S proteasomes and the ATP-ubiquitin proteolytic pathway in

25 the generation of antigenic peptides for MHC-1 antigen presentation. In addition, this work reveals new features of the pathway of proteolysis of antigens and of proteasome function, which led to the present invention. Firstly, they have shown that gamma

30 interferon, which is known to stimulate antigen presentation, selectively enhances those peptidase activities, namely, the trypsin-like and chymotrypsin-like activities, of the proteasomes that produce

-10-

peptides that can bind to MHC-1 molecules, i.e. peptides with basic and hydrophobic carboxyl-terminal residues (Example 1). Gamma interferon increased 2- to 6-fold the capacity of purified 20S and 26S proteasomes to  
5 cleave peptides after hydrophobic and basic residues, while reducing cleavage after acidic residues. They have further shown that this enhancement by gamma interferon is dependent on the expression of the MHC gene products, LMP 2 and LMP 7. Their findings, thus,  
10 provide evidence to support the role of proteasomes in antigen presentation. Furthermore, they indicate that the three peptidases of proteasomes are distinctly regulated, that changes in the relative activities of the peptidases can alter the nature of peptides that are  
15 generated by the proteasome and which are available for MHC-1 antigen presentation, and that the MHC-encoded subunits are involved in the regulation of the peptidase activities.

Secondly, Applicants have shown that a defect in  
20 ubiquitin conjugation, an early step in the ATP-ubiquitin-dependent proteolytic pathway, leads to reduced MHC-I-restricted antigen presentation (Example 2). Using cells exhibiting a temperature-sensitive defect in the ubiquitin activation enzyme, E1, they  
25 found that the nonpermissive temperature inhibited class I-restricted presentation of ovalbumin (OVA) introduced into the cytosol, but did not affect presentation of an OVA peptide synthesized from a minigene.

These findings provide evidence for the role of the  
30 ATP-ubiquitin proteolytic system in MHC-I antigen presentation. They demonstrate that a block at either an early step (ubiquitin conjugation) or a late step (processing of carboxyl-termini of the peptides) in the

-11-

proteolysis of intracellular proteins by the ATP-ubiquitin system can inhibit class I restricted presentation. It is reasonable to expect that inhibition of other steps in this proteolytic pathway  
5 will also produce a similar effect.

Applicants' discoveries of these features of proteasome function and their demonstration that inhibition at a single step in the ATP-ubiquitin pathway can inhibit antigen presentation lead to the present  
10 invention. It is reasonable to expect that inhibitors that block single or multiple steps of this proteolytic pathway would inhibit MHC-I-restricted antigen presentation, and that such inhibitors would be useful in reducing CD8<sup>+</sup> mediated cytolytic immune responses.  
15 Such inhibitors should be useful for therapy or prevention of autoimmune diseases and for reducing rejection of transplanted and other foreign tissues. Inhibitors of either or both the trypsin-like and chymotrypsin-like peptidases of proteasomes and  
20 inhibitors of ubiquitin conjugation are particularly expected to be useful for these purposes.

As described herein, Applicants have demonstrated the feasibility of this invention by showing that chymostatin, which selectively inhibits cleavage after  
25 hydrophobic residues, inhibits the MHC-I presentation of ovalbumin (Example 3). Antigen presentation of an OVA peptide introduced into the cytosol was not inhibited, indicating that chymostatin is affecting the processing of the OVA protein into the peptide. This and other  
30 inhibitors are further described in the following section.

-12-

Inhibitors of the ATP-Ubiquitin Dependent Proteolytic Pathway

Various inhibitors of the peptidases of proteasomes have been reported (see, e.g., Tanaka et al., 1992; 5 Orłowski, 1990; Goldberg, 1992; Rivett et al., 1989a [Arch. Biochem. Biophys. 268:1-8] and 1989b [J. Biol. Chem. 264:12,215-12219]; Dick et al., 1991). These include known inhibitors of chymotrypsin-like and trypsin-like proteases, as well as inhibitors of thiol 10 (or cysteine) and serine proteases. In addition, some endogenous inhibitors of proteasome activities have been isolated. These include the 240 kD and the 200 kD inhibitors isolated from human erythrocytes (Murakami and Etlinger, 1986; Li et al., 1991 and purified CF-2 15 (described in Example 4; Goldberg, 1992). As exemplified by these known inhibitors of proteasomes, other molecules, including known inhibitors of chymotrypsin-like, trypsin-like, thiol, and serine proteases, can be tested using assays for the proteasome 20 peptidases (see Examples).

The inhibitors can be naturally isolated or synthetic and peptide or non-peptide molecules. Preferably, the inhibitors would selectively inhibit the chymotrypsin-like and trypsin-like peptidases, while 25 leaving the peptidylglutamyl peptidase relatively unaffected. A typical inhibitor would be a peptide aldehyde, like leupeptin, which inhibits primarily cleavage after basic residues, or chymostatin, which inhibits primarily after hydrophobic residues. In 30 addition to these antibiotic inhibitors originally isolated from Actinomycetes (Aoyagi and Umezawa, 1975), a variety of peptide aldehydes have been synthesized,

-13-

such as the inhibitors of chymotrypsin-like proteases described by Siman et al. (WO 91/13904).

Novel molecules can also be obtained and tested for inhibitory activity. As illustrated by the above cited references, various strategies are known in the art for obtaining the inhibitors for a given protease. Compound or extract libraries can be screened for inhibitors using peptidase assays. Alternatively, peptide and peptidomimetic molecules can be designed based on knowledge of the substrates of the protease. For example, substrate analogs can be synthesized containing a reactive group likely to interact with the catalytic site of the protease (see, e.g., Siman et al., WO 91/13904; Powers et al., 1986). The inhibitors can be stable analogs of transition intermediates (transition state analogs); such as Cbz-Gly-Gly-leucinal, which inhibits the chymotrypsin-like activity of the proteosome (Orlowski, 1990; see also Kennedy and Schultz, 1979). Variants or analogs of known inhibitors, such as chymostatin, can be also be synthesized.

Various natural and chemical protease inhibitors reported in the literature, or molecules similar to them, are likely to inhibit the activity of the two proteasomal peptidases. These include peptides containing an  $\alpha$ -dicarbonyl unit, such as an  $\alpha$ -diketone or an  $\alpha$ -keto ester, peptide chloromethyl ketones, isocoumarins, peptide sulfonyl fluorides, peptidyl boronates, peptide epoxides and peptidyl diazomethanes (Angelastro et al., 1990; Bey et al., EPO 363,284; Bey et al., EPO 364,344; Grubb et al., WO 88/10266; Higuchi et al., EPO 393,457; Ewoldt et al., 1992; Hernandez et al., 1992; Vlasak et al., 1989; Hudig et al., 1991;

-14-

Odakc et al., 1881; Vijayalakshmi et al., 1991; Kam et al., 1990; Powers et al., 1989; Oweida et al., 1990; Powers et al., 1990; Hudig et al., 1989; Orłowski et al., 1989; Zunino et al., 1988; Kam et al., 1988; Parkes et al., 1985; Green and Shaw, 1981; Angliker et al., 1987; Puri et al., 1989; Hanada et al., 1983; Kajiwara et al., 1987; Rao et al., 1987; Tsujinaka et al., 1988].

Various inhibitors of ubiquitin conjugation to proteins are also known (see Wilkinson et al., 1990).

10 Ubiquitin conjugation itself is a multistep process, involving the activities of an ubiquitin activating enzyme (E1), a group of ubiquitin-carrier proteins (E2), which catalyze the transfer of ubiquitin to target proteins, and the ubiquitin-protein ligase, E3.

15 Molecules that block ubiquitin conjugation at any of these steps are also expected to be inhibitors of antigen presentation. Preferably, specific inhibitors of these enzymes will be used, such as substrate and transition state analogs. For example, adenosyl-

20 phospho-ubiquitinol, an analog of the substrate ubiquitin adenylate, has been found to be a specific and effective inhibitor of E1 (Wilkinson et al., 1990).

The inhibitors can be used *in vitro* or *in vivo* to block MHC-I antigen presentation. They can be

25 administered by any number of known routes, including orally, intravenously, intramuscularly, topically, and by infusion (see, e.g., Platt and Stracher, U.S. Patent 4,510,130; Badalamente et al., 1989; Staubli et al., 1988). Preferably, the inhibitors are low molecular

30 weight molecules. Suitable vehicles for protein drug delivery, such as liposomes, may also be used. Vehicles than can target the drug to specific tissues can also be used.



-15-

Examples

The following Examples further describe Applicants' work in more detail and more specifically illustrate this invention. Tables are located at the end of each Example.

Example 1Regulation of the Peptidase Activities of Proteasomes  
By  $\gamma$ -IFN and MHC Genes

The present studies were undertaken to clarify the role of  $\gamma$ -IFN, proteasomes, and MHC-encoded proteins in the processing of cell antigens. The work described below demonstrates that the pattern of peptidase activities of the 20S and 26S proteasome complexes is altered following treatment of cells with  $\gamma$ -IFN and upon expression of the MHC region that contains the LMP 2 and 7 genes. These changes in catalytic activities should favor the generation of the types of peptide sequences (Falk et al., 1991) that bind specifically to MHC-class I proteins.

 $\gamma$ -Interferon Alters Proteosome Activities.

To learn whether  $\gamma$ -IFN regulates the function of proteasomes, several hydrolytic activities of the proteasomes and of soluble extracts from control U937 cells (a human monocytic line) and U937 cells treated with  $\gamma$ -IFN were examined. Although this treatment caused a large induction of MHC-class I genes and proteins encoded in the MHC region (i.e. LMP 2 or TAP 1), no significant difference was found in the ability of isolated proteasomes or cell extracts to degrade  $^{125}\text{I}$ -lactalbumin or ubiquitinated  $^{125}\text{I}$ -lactalbumin to

-16-

acid-soluble fragments. However, clear changes were found after  $\gamma$ -IFN treatment in the ability of the proteasomes to hydrolyze different peptide substrates. Fluorogenic peptide substrates were used to measure the hydrolytic activities of proteasomes: Suc-LLVY-MCA [SEQ ID No: 3] (hydrophobic) for the "chymotrypsin-like" peptidase, Boc-LRR-MCA (basic) for the "trypsin-like" peptidase, and Cbz-LLE-MNA (acidic) for the peptidylglutamyl peptidase.

Proteasome-enriched fractions, as well as crude extracts, from  $\gamma$ -IFN-treated U937 cells hydrolyzed the hydrophobic peptide, Suc-LLVY-MCA, 2- to 3-fold faster and the basic peptide, Boc-LRR-MCA, 3- to 4-fold faster than similar preparations from control cells (Figure 1). These effects of  $\gamma$ -IFN-treatment were seen in the absence of ATP. Kinetic analysis indicated that  $\gamma$ -IFN caused a 2-fold increase in the maximal capacity ( $V_{\max}$ ) of the proteasome to hydrolyze the hydrophobic substrate and a 6-fold increase in  $V_{\max}$  with the basic substrate (Figure 1). In addition, a small increase in the  $K_m$ s for these substrates (averaging 50%) was found consistently, but such an effect cannot account for the accelerated peptide hydrolysis (Figure 1). Very similar changes in  $V_{\max}$  were seen in analogous studies using purified 20S proteasome (see below). The cleavage of a variety of other hydrophobic and basic peptides, which are degraded more slowly than Suc-LLVY-MCA or Boc-LRR-MCA (respectively), increased in a similar fashion after  $\gamma$ -IFN treatment, as did hydrolysis of these standard substrates (Table 1). Thus, proteasomes from  $\gamma$ -IFN-treated cells are generally more efficient in cleaving after hydrophobic and basic residues, although the

-17-

specificities of these active sites do not appear to change.

Under these conditions, the overall capacity of the cells to hydrolyze these basic and hydrophobic peptides increases. However, no change was found in total proteasome content. The ability of the crude extracts to hydrolyze these peptides changed in a similar fashion, as did proteasome fractions, which contain about 80% of the activities against these substrates in post-nuclear cell extracts. Most of the remaining peptidase activities (15% total) was due to proteasomes that were tightly associated with microsomal membranes, and  $\gamma$ -IFN treatment also enhanced the cleavage of Suc-LLVY-MCA and Boc-LRR-MCA by proteasomes in the microsomal and nuclear fractions.

It is noteworthy that not all of the proteasomes's peptidase activities increase after  $\gamma$ -IFN treatment. Proteasomal fractions and the crude extracts from  $\gamma$ -IFN-treated U937 cells cleaved the acidic substrate, Cbz-LLE-MNA (at 100 mM), at roughly similar rates as controls. Further kinetic analysis, however, indicated that there was a reproducible decrease of approximately 30% in the  $V_{\max}$  value for this substrate (Figure 1) (as well as a decrease in the  $K_m$ ). Thus, the several catalytic sites of the proteasome are regulated in distinct fashions by  $\gamma$ -IFN.

#### Behavior of 20S and 26S Complexes

Within cells, proteasomes are found either as part of the 26S (1,500 kD) complexes, which degrade preferentially ubiquitin-conjugated proteins, or as 20S particles, which by themselves cannot hydrolyze Ub-conjugated proteins, despite having multiple proteolytic

-18-

activities (Goldberg and Rock, 1992; Goldberg, 1992; Tanaka et al., 1992; Armon et al., 1990; Driscoll and Goldberg, 1992; Eytan et al., 1989; Driscoll and Goldberg, 1989; Matthews et al., 1989). To examine whether  $\gamma$ -IFN alters the catalytic activities of both the 20S and 26S proteasomes, these structures were purified by FPLC using anion exchange and gel filtration columns (Figure 2). A good separation of the 20S and 26S forms of the proteasome was achieved. Native PAGE of the 20S fraction showed the characteristic 700 kD band, while the 20S samples contained a 1500 kD band plus some 700 kD material. As expected, these 26S preparations degraded ubiquitinated- $^{125}$ I-lactalbumin in the presence of ATP, but exhibited little or no activity against free  $^{125}$ I-lactalbumin. In contrast, the 20S fraction degraded this protein, but not if it was ubiquitinated. Both 20S and 26S forms exhibited the three peptidase activities, which could be stimulated several-fold by ATP. ATP (2 mM) stimulated the degradation of Suc-LLVY-MCA by 20S proteasomes and the degradation of the same substrate 5-fold by 26S proteasomes from control cells. In the presence of ATP, significant differences between control and  $\gamma$ -IFN-treated preparations were retained for 20S proteasomes..

Interferon-treatment caused similar changes in the peptidase activities of both 20S and 26S proteasomes, as were found with cruder preparations. With both forms, cleavages of hydrophobic and basic substrates increased significantly with  $\gamma$ -IFN, although the percent increase tended to be somewhat smaller for the 26S fraction. Interestingly, cleavage of the acid substrate by either particle did not increase, and its hydrolysis by the 26S particle decreased by 50% after  $\gamma$ -IFN treatment (Figure

-19-

2). By contrast, after  $\gamma$ -IFN treatment, no consistent differences were observed in the abilities of the 20S or 26S particles to degrade free  $^{125}\text{I}$ -lactalbumin or ubiquitinated  $^{125}\text{I}$ -lactalbumin either in the absence or  
5 presence of ATP. Taken together, these findings suggest that  $\gamma$ -IFN alters the pattern of cleavages in polypeptides made by both 20S and 26S proteasomes, without affecting the overall rates of protein digestion.

#### 10 **Proteasome Activities in MHC-Deficient Cells**

Since two proteins encoded in the MHC region, LMP 2 and LMP 7, associate with proteasomes (Driscoll and Finley, 1992; Goldberg and Rock, 1992; Monaco and McDevitt, 1986; Parham, 1990), and since  $\gamma$ -IFN  
15 stimulates the expression of these components, Applicants investigated whether deletion of this part of the MHC-locus might influence the peptidase activities of proteasomes and their response of  $\gamma$ -IFN. For this purpose, they isolated the proteasomal fraction from 721  
20 human B lymphoblastoid cells and from 721.174, a variant of these cells, that carries a homozygous deletion in the MHC region that includes the LMP 2 and LMP 7 genes (DeMars et al., 1985). Proteasomes from the mutant cells were found to degrade the basic substrate at  
25 approximately 40% of the rate and the hydrophobic substrate at 70% of the rate of the wild-type cells (Figure 3).  $V_{\max}$  values for the two substrates decreased by more than 50% for proteasomes from mutant cells (Figure 3). The total content of the proteasomes  
30 appeared similar in the two cell types, and no clear difference was seen in the ability of the proteasomes to degrade  $^{125}\text{I}$ -lysozyme or ubiquitinated- $^{125}\text{I}$ -lysozyme to

-20-

acid-soluble products. Furthermore, the breakdown of several more slowly degraded hydrophobic and basic substrates (the same ones studied in Table 1) was also lower in the MHC-deleted mutant by 40% to 80. In contrast to the reduction in these peptidase activities, proteasomes from the MHC-deleted variant consistently cleaved the acidic substrate, Cbz-LLE-MNA, about 40% faster than similar preparations from wild-type cells (Figure 3). A small, but consistent, increase in the  $V_{\max}$  was seen for this substrate (Figure 3).

Similar changes in the three peptidase activities were found in crude extracts from the mutant and wild-type cells and proteasome microsomal or "nuclear" fractions. Thus, the peptidase capacity of proteasomes in all fractions of the cell appear to be regulated by MHC-encoded genes and by  $\gamma$ -IFN. The findings that expression of MHC-encoded genes increases basic and hydrophobic cleavages by the proteasome, while suppressing acidic cleavages, parallels the results obtained when U937 cells were treated by  $\gamma$ -IFN. These observations raise the possibility that the  $\gamma$ -IFN-dependent changes in proteasome activities are mediated, at least in part, through the expression of MHC-encoded proteins, most likely the LMP 2 and LMP 7 proteasome subunits. To examine this hypothesis, Applicants used the 721 and 721.174 variant to investigate whether  $\gamma$ -IFN treatment could alter these peptidase activities in the absence of the LMP genes.

In wild-type cells treated with  $\gamma$ -IFN, there was an increased rate of degradation of hydrophobic and basic substrates and a reduced capacity to cleave the acidic substrate. Thus, the proteasomes from wild-type lymphocytes responded in a manner similar to those of

-21-

U937 cells (although the relative changes appeared larger in the monocyte line). However,  $\gamma$ -IFN treatment of the MHC-deleted cells did not alter at all the degradation of the basic substrate, Boc-LRR-MCA, in contrast to the large changes seen with proteasomes from wild-type cells. In the 721.174 mutant,  $\gamma$ -IFN caused a much smaller increase in cleavage of the hydrophobic peptide, Suc-LLVY-MCA, than in the wild-type parent cells. These differences between the cells were evident with a number of different basic and hydrophobic peptides (Table 2). Surprisingly, in mutant lymphoblasts,  $\gamma$ -IFN actually enhanced hydrolysis by proteasomes of the acidic substrate (Table 2), even though in the wild-type parent cells (and in the U937 line),  $\gamma$ -IFN reduced reproducibly cleavage of this substrate. Clearly, the IFN-induced alterations in the activities of the three catalytic sites of the proteasome, either directly or indirectly, are MHC-dependent.

## 20 Protein Breakdown and Proteasome Function

Two general new conclusions have emerged from these experiments: 1) that the catalytic functions of proteasomes are qualitatively regulated by gamma-interferon and these actions require the presence of MHC-encoded genes; and 2) that the different peptidase sites of these particles can be regulated in distinct fashions. After  $\gamma$ -IFN treatment, proteasomes in monocytes and lymphoblasts, and presumably in many other cells, show a greater capacity to hydrolyze peptides following hydrophobic and basic residues, but a reduced capacity to cleave after acidic residues.

-22-

Previous studies have demonstrated several cellular factors that can bind reversibly to the proteasome and stimulate (Dubiel et al., 1992; Ma et al., 1992b) or inhibit (Driscoll et al., 1992; Li et al., 1991) its  
5 three peptidase activities coordinately (Goldberg and Rock, 1992; Goldberg, 1992; Tanaka et al., 1992). In contrast,  $\gamma$ -IFN affects each of these catalytic sites differently, presumably because it regulates gene expression and thereby alters the subunit composition of  
10 the proteasome (see below). Interestingly,  $\gamma$ -IFN was not found to alter the rates of digestion of a protein substrate,  $^{125}\text{I}$ -lactalbumin or ubiquitin-conjugated  $^{125}\text{I}$ -lactalbumin to acid-soluble fragments by either the 20S or 26S proteasome. These findings suggest that *in vivo*,  
15 the initial, presumably rate-limiting, cleavages of polypeptides or ubiquitinated proteins by proteasomes are not altered by  $\gamma$ -IFN. Instead, this cytokine appears to affect the later steps in this pathway, i.e. the subsequent cleavages of the acid-soluble protein  
20 fragments to short peptides. Thus, the changes in the relative activities of the three peptidases should alter the nature of the oligopeptides that are generated by the proteasome and are available for further proteolytic processing and/or delivery to MHC-class I molecules (see  
25 below).

It will be important to define the structural changes in the protease that lead to the increased rates of hydrolysis after basic and hydrophobic sequences and to the decreased cleavage after acidic residues. The  
30 major alterations in these activities induced by  $\gamma$ -IFN clearly require the presence of the portion of the MHC region containing the LMP 2 and 7 genes. Deletion of this region by itself (without  $\gamma$ -IFN treatment) caused



-23-

the reverse changes in peptidase activities of those induced by  $\gamma$ -IFN. Furthermore,  $\gamma$ -IFN is known to stimulate the expression of the two MHC-encoded subunits, LMP 2 and LMP 7, and to increase the abundance of "LMP particles" in cells (i.e. those proteasomes containing LMP subunits). The simplest explanation for these findings is that the  $\gamma$ -IFN-induced alterations in catalytic properties result from incorporation into proteasomes of these MHC-encoded subunits. The LMP 2 and/or LMP 7 proteins may themselves contain "tryptic-like" or "chymotryptic-like" sites. If so, these sites may supplement the peptidase sites present in the absence of  $\gamma$ -IFN or may replace them with sites of a higher catalytic efficiency. In this content, it is intriguing that, in yeast, the "chymotryptic-like" activity of proteasomes is inactivated by point mutations in a proteasome-subunit gene very homologous to LMP 2 (Driscoll et al., 1992; Li et al., 1991; Ma et al., 1992). Alternatively, incorporation into proteasomes of LMP 2 or LMP 7 might enhance the activity of other subunits that contain the trypsin-like or chymotrysin-like catalytic sites. Similarly, incorporation of these MHC-encoded subunits may replace or reduce the activity of subunits that comprise the peptidylglutamyl catalytic site of the proteasomes.

Although these findings suggest functions for the LMP-encoded subunits,  $\gamma$ -IFN clearly induce some changes in proteasome activity in the mutants lacking LMP 2 and LMP 7 genes. In these cells,  $\gamma$ -IFN caused a small (but reproducible) increase in the chymotryptic activity. In addition, the peptidylglutamyl activity in the mutants increases upon  $\gamma$ -IFN treatment, even though in wild-type cells, this activity decreases. These functional

-24-

changes must arise through other alterations in proteasome subunit composition, not involving LMP 2 and LMP 7. Presumably,  $\gamma$ -IFN can reduce or suppress the synthesis of subunits encoded outside of this portion of the MHC region or may otherwise modify proteasome structure. In fact, Peterson and coworkers (Yang et al., 1992) have reported that  $\gamma$ -IFN alters the pattern of proteasome subunits in mutants lacking the LMP genes.

It is noteworthy that LMP 2 and LMP 7 proteins are present in only a subset of the cell's proteasomes (Brown et al., 1991), and within this subset, some particles may exist that contain only one of these subunits, while others may contain both. In either case, the LMP 2- and LMP 7-containing populations probably differ to a very large degree from the preexistent population in their hydrolytic activities, since the induction of these subsets or proteasomes by  $\gamma$ -IFN were observed to cause large changes in the total peptidase activities in the cell within 3 days. Thus, the changes in catalytic activity found here probably underestimate the actual differences between the activities of LMP-containing and LMP-deficient subsets of proteasomes.

In the past, the proteasome has generally viewed as a single type of structure with characteristic enzymatic properties. This view is clearly simplistic or incorrect in light of the present findings, which emphasize the functional heterogeneity and plasticity of these particles. These findings suggest that in many cells, proteasome properties change in vivo during infections or sepsis or other conditions, in which  $\gamma$ -IFN levels rise and immune responses are activated. These alterations in proteasome properties must depend also on

-25-

dosage and duration of exposure to this cytokine. The existence of functionally distinct subclasses of proteasomes in cells may also be important in many other biological contexts. In fact, there have been reports  
5 of alterations in proteasome-subunit composition during cell development (Ahn et al., 1991; Scherrer, 1990), which presumably result in distinct changes in function and have different physiological consequences from those reported here.

#### 10 **Implications for Antigen Presentation**

As part of normal protein turnover, most of the peptides generated by the proteasomes in cytosol or nucleus must be rapidly digested to free amino acids by non-proteasomal peptidases. However, a fraction appears  
15 to be transported into the endoplasmic reticulum (ER) by the TAP 1-2 complex and to bind to MHC-class I molecules, either directly or after further proteolytic processing (Townsend and Bodmer, 1989; Yewdell and Bennink, 1992; Monaco, 1992; Powis et al., 1991; Spies  
20 and DeMars, 1991). The changes in the proteasomal activities induced by  $\gamma$ -IFN or resulting from the MHC-deletion should alter the peptides generated by the proteasome during degradation of cellular and viral proteins, and thus change the repertoire of peptides  
25 available for presentation to T cells. These alterations in the relative rates of cleavage after basic, hydrophobic, and acidic residues should result in production of a different group of peptides or of the same peptides, but in very different proportions.  
30 Specifically, the 2- to 6-fold increase in the "chymotryptic-like" and "tryptic-like" activities and the decrease in peptidylglutamyl activity should

-26-

generate more short peptides ending with basic or hydrophobic carboxyl termini, and fewer peptides ending with acidic carboxyl termini.

These changes in catalytic properties with  $\gamma$ -IFN or expression of MHC-encoded genes would thus appear to favor the generation of just those types of peptides that are known to bind to MHC-class I molecules. The C-terminal residue of antigenic peptides plays a key role in their binding to specific MHC-class I molecules (Falk et al., 1991; Guo et al., 1992; Hunt et al., 1992; Jardetzky et al., 1991; Matsumura et al., 1992; Parham, 1992), as is also evident from the X-ray analysis of several MHC-protein-peptide complexes (Rotzschke and Falk, 1991; Fremont et al., 1992; Silver et al., 1992; Zhang et al., 1992). It is indeed striking that the vast majority of naturally-processed peptides that have been found associated with class I molecules have hydrophobic or basic C-termini, and virtually none has an acidic C-terminus (Figure 4). This conclusion is based upon Applicants' survey of the published literature of MHC-associated peptides. Furthermore, an even stronger preference for hydrophobic C-termini is evident in the sequences of the average population of peptides that can be eluted from two specific subtypes of MHC-class I proteins. This predominance of basic and hydrophobic residues was not seen at the amino acids preceding the N-termini of MHC-associated peptides (Figure 4).

Several immunological roles have been postulated for LMP 2 and LMP 7 subunits. It has been proposed that the LMP subunits direct the proteasome particles to the endoplasmic reticulum (ER), where they would be in contiguity with the TAP-1/2 transporters. However,

-27-

Applicants and others (Yang et al., 1992) have found no evidence that the loss of LMP genes or  $\gamma$ -IFN-stimulation of LMP expression affects the subcellular distribution of proteasomes. Instead, Applicants have found that the  
5  $\gamma$ -IFN-treatment and MHC-deletion change the peptidase activities of proteasomes in the soluble fraction of the cell, as well as the activities of the minor population of proteasomes associated with ER (10% total) and with the nuclear fraction. For transport into the ER, these  
10 antigenic peptides generated by soluble proteasomes must somehow withstand complete hydrolysis by cytosolic exopeptidases.

More recently, it has been argued that LMPs do not play an important role in antigen presentation (Arnold  
15 et al., 1992; Mamburg et al., 1992), because MHC-deleted cells (like 721.174) can present antigens if they are transfected only with the genes for the TAP-1 and -2 transporters. This work shows, however, that the proteasomes isolated from these mutant cells still  
20 exhibit the tryptic- and chymotryptic-like activities. These findings may explain why the MHC-deleted cells can still present antigens on class I molecules. The results lead to the prediction of a decrease in the efficiency of antigen processing and presentation by the  
25 MHC-deleted mutant cells. This was in fact reported by Hammerling and coworkers (Mamburg et al., 1992). On the other hand, the actions of  $\gamma$ -IFN to increase expression of LMPs and thereby to change the pattern of peptides produced by the proteasome may contribute, along with  
30 the induction of the transporters and MHC-class I proteins, to the enhancement of antigen presentation.

-28-

### Methods

#### **Treatment of Cells with $\gamma$ -IFN**

U937 cells ( $0.15 \times 10^6/\text{ml}$ ) were grown in  $150 \text{ cm}^3$  flasks, in RPMI 1640 medium containing 10% FCS and antibiotics at  $37^\circ\text{C}$  for 72 hours in the presence or absence of 1000 U/ml of human recombinant  $\gamma$ -IFN (kindly provided by Biogen, Inc. Cambridge, MA). In preliminary studies, this concentration of  $\gamma$ -IFN was found to cause maximal changes of proteasomal peptidases activities in U937 cells. Mutant (721.174) and wild-type (721) cells were grown for three days in the presence or absence of 3,000 U/ml of  $\gamma$ -IFN, which was found to cause maximal changes in peptidases activities.

#### **Peptidase Assays**

Hydrolysis of  $^{125}\text{I}$ -human lactalbumin or ubiquitin-conjugated- $^{125}\text{I}$ -lactalbumin was assayed by measuring the production of radioactive peptides soluble in 10% trichloroacetic acid (Waxman et al., 1987). Proteasome fractions or crude extracts ( $0.1 \text{ mg/ml}$ ) and purified proteasomes ( $5 \text{ }\mu\text{g/ml}$  of 20S or  $15 \text{ }\mu\text{g/ml}$  of 20S); were incubated in the Buffer B (see Detailed Description of Figure 1) with radioactive substrates at  $37^\circ\text{C}$  for 60 or 120 minutes in the presence of 2 mM ATP or 5 U/ml of apyrase (to destroy residual ATP). Nonradioactive lactalbumin ( $20 \text{ }\mu\text{g/ml}$ ) was added to reaction mixtures containing Ub- $^{125}\text{I}$ -lactalbumin.

#### **Preparation of Crude Cell Extracts and Proteasome-Enriched Fractions**

To prepare extracts, cells were collected by centrifugation for 5 minutes at  $700 \times g$ , washed twice, and resuspended in Homogenization Buffer (50 mM Tris, 5

-29-

mM  $\text{MgCl}_2$ , 1 mM dithiothreitol (DTT), 2 mM ATP, 250 mM sucrose, pH 7.4). Cell suspensions were homogenized by several passages through a Dounce homogenizer (Wheaton), followed by vortexing for 3 minutes with glass beads.

5 After centrifugation at 10,000 x g for 20 minutes, the supernatant ("crude extract") was centrifuged for 1 hour at 100,000 x g to obtain a "microsome pellet". The "proteasome fraction" was obtained by spinning the post-microsomal supernatant for 5 hours at 100,000 x g.

10 Pellets were resuspended in about 10 volumes of Buffer A (50 mM Tris, 5 mM  $\text{MgCl}_2$ , 1 mM DTT, 2 mM ATP, 20% glycerol), homogenized and centrifuged at 14,000 rpm for 15 minutes. The supernatants, which contained 20% of the protein in crude extracts, were adjusted to a

15 concentration of 1 mg/ml (Bradford, 1976) prior to assay of proteolytic activities, as were the crude extracts (after dialysis against Buffer A). With each set of cultured cells, two sets of extracts were prepared and analyzed in parallel. The averages of results obtained

20 with the two sets were taken as the result of a particular experiment.

#### Detailed Description of Figure 1

The upper panel of Figure 1 shows the hydrolysis of the indicated substrates (100  $\mu\text{M}$ ) by proteasome

25 fractions from control cells and cells treated with  $\gamma$ -IFN (Mamburg et al., 1992). Peptidase activities were determined by the release of 7-amino-4-methylcoumarin (MCA) or methoxynaphthylamine (MNA) from fluorogenic peptide substrates (Ischiura et al., 1985). Proteasome

30 fractions (final protein concentration: 50  $\mu\text{g}/\text{ml}$  in 0.1 ml of Buffer B: 50 mM Tris, 5 mM  $\text{MgCl}_2$ , 5 mM DTT, pH 7.4, containing 5 U/ml apyrase) were incubated for 40 or

-30-

60 minutes at 37°C. The reaction was terminated by adding 1 ml of 1% sodium dodecyl sulphate. The increase in degradation of hydrophobic and basic substrates was highly significant ( $P < 0.01$  with  $n=6$  different experiments). In the crude extracts, very similar changes ( $P < 0.02$ ,  $n=3$ ) were observed with Suc-LLVY-MCA and Boc-LRR-MCA, while degradation of Cbz-LLE-MNA did not change significantly. Units = mM of substrate cleaved per mg of protein per hour.

10 The lower panel of Figure 1 shows kinetic analysis (Lineweaver-Burk method) of rates of cleavage of the substrates (v) by the same proteasome fractions used above. Incubations were carried out as in upper panel, with varying substrate concentration (S).  $V_{max}$  for Suc-LLVY-MCA was 0.7 units and 1.6 with  $\gamma$ -IFN; for Boc-LRR-MCA, 0.3 units and 1.9 with  $\gamma$ -IFN; for Cbz-LLE-MNA, 0.3 units and 0.2 with  $\gamma$ -IFN.  $K_m$  for Suc-LLVY-MCA was 0.2 mM and 0.3 mM with  $\gamma$ -IFN; for Boc-LRR-MCA, 0.4 mM and 0.5 mM with  $\gamma$ -IFN; for Cbz-LLE-MNA, 0.10 mM and 0.06 mM  
15  
20 with  $\gamma$ -IFN.

#### Detailed Description of Figure 2

Figure 2 shows the effect of  $\gamma$ -IFN treatment on peptidase activities in 20S and 26S proteasomes purified from U937 cells. The results are representative of 3  
25 different experiments. All differences between control preparations and those from  $\gamma$ -IFN-treated cells were statistically significant ( $P < 0.01$ ), but no significant difference was seen for Cbz-LLE-MNA cleavage by 20S. Units and assay conditions are as in Figure 1, but the  
30 final concentrations of the proteasomes were 2.5-3.0  $\mu$ g/ml (26S) or 8-10  $\mu$ g/ml (20S). 20S and 26S proteasomes were isolated from the proteasome fraction



-31-

by Q Sepharose anion exchange and Superose 6 gel filtration FPLC chromatographies (Pharmacia) (Driscoll and Goldberg, 1989; Matthews et al., 1989). The 26S-rich fraction was identified by its ability to support the ATP-dependent degradation of Ub-<sup>125</sup>I-lactalbumin. Routinely, about 30 µg of 26S and 90 µg of 20S were obtained from 5 mg of proteasome fraction.

#### Detailed Description of Figure 3

The upper panel of Figure 3 shows the difference in activities of the peptidases of proteasomes from wild-type (721) and MHC-deficient (721.174) lymphoblastoid cells. The rates of hydrolysis of three substrates (100 µM) were measured in proteasome fractions, as done for Figure 1. Differences in all three activities were statistically significant ( $P < 0.01$ ,  $n = 4$  different preparations).

The lower panel of Figure 3 shows the kinetic analysis of cleavage for all the substrates at different concentrations (S) by the same proteasome fractions from wild-type and mutant cells.  $V_{max}$  for Suc-LLVY-MCA was 6.5 units (wild-type) versus 2.4 (mutant); for Boc-LRR-MCA, 1.6 units vs 0.7; for Cbz-LLE-MNA, 0.7 units vs 1.2.  $K_m$  for Suc-LLVY-MCA was 0.5 mM (wild-type) vs 0.3 mM (mutant); for Boc-LRR-MCA, 0.3 mM vs 0.5 mM; for Cbz-LLE-MNA, 0.4 mM (wild-type and mutant). Very similar results were obtained in two different experiments.

#### Detailed Description of Figure 4

The left panel of Figure 4 shows the frequency of amino acids at the carboxyl termini of 44 peptides that bind MHC class I molecules. The data were collected from published sequences of peptides (Falk et al., 1991;

-32-

Guo et al., 1992; Hunt et al., 1992; Jardetzky et al., 1991; Matsumura et al., 1992). These sequences were determined in different studies either by Edman degradation of individual peptides that were eluted and  
5 purified from MHC class I molecules or by functional analysis of synthetic antigenic peptides and alignment to known MHC-binding motifs. The MHC class I molecules included in this analysis fall into two groups:  $K^b$ ,  $D^b$ ,  $K^d$ ,  $L^d$  and HLA-A2.1, which bind peptides that generally  
10 have a hydrophobic C-terminal residue, and HLA-B27 and HLA-AW68, which bind peptides that generally have a basic C-terminal residue.

The right panel of Figure 4 shows the frequency of amino acids preceding the N-termini of MHC class I-bound  
15 peptides. The protein sequences from which 39 of the peptides in the left panel were derived were available. Data were collected from the EMBL (Heidelberg, Germany) and National Biomedical Research Foundation (Bethesda, Md) databases.

-33-

TABLE 1

Stimulation of the Trysin-like and Chymotrysin-like  
Peptidases of Proteasomes by  $\gamma$ -IFN

Substrate	Activity ( $\mu$ M cleaved/mg/h)		
	Control	+ $\gamma$ -IFN	% increase
<u>Hydrophobic</u>			
Suc-LLVY-MCA	230	820	+357
Suc-LY-MCA	90	210	+133
Suc-AAF-MCA	60	120	+100
<u>Basic</u>			
Boc-LRR-MCA	57	250	+439
Boc-GGR-MCA	3.4	5.2	+53
Cbz-GKR-MCA	2.8	6.0	+114

Degradation rates of substrates (100  $\mu$ M) by proteasome fractions of U937 cells were analysed as in Figure 1 (upper panel). Presented are averages from 6 (Suc-LLVY-MCA and Boc-LRR-MCA) or 2 (all other substrates) different experiments.

-34-

TABLE 2

Comparison of  $\gamma$ -IFN-Induced Changes in Peptidase Activities of Proteasomes from Wild-type and MHC-deficient Cells

Substrates	<u><math>\gamma</math>-IFN-induced Increase in Activity</u>			
	Wild-type		MHC-deficient Mutant	
	units <sup>1</sup>	(% change)	units <sup>1</sup>	(% change)
<u>Hydrophobic</u>				
Suc-LLVY-MCA	340	(+33)	120	(+18) <sup>†</sup>
Suc-LY-MCA	66	(+34)*	10	(+26) <sup>†</sup>
Suc-AAF-MCA	62	(+42)*	8	(+15) <sup>†</sup>
<u>Basic</u>				
Boc-LRR-MCA	180	(+48)*	ns	
Boc-GKR-MCA	1.6	(+28)*	ns	
Cbz-GGR-MCA	1.2	(+34)*	ns	
<u>Acidic</u>				
Cbz-LLE-MNA	-40	(-24)*	+80	(+35) <sup>†</sup>

Wild-type cells were 721 and mutant cells were 721.174. With all hydrophobic and basic substrates,  $\gamma$ -IFN caused consistently (n=3) larger changes in the wild-type cells. Interferon consistently caused opposite effects with the acidic substrate in the wild-type and mutant cells. Data are differences between means for proteasome fractions. Activities were determined as in Figure 1.

<sup>1</sup> Units =  $\mu$ M of substrate cleaved per mg of protein per hour.

\* P<0.02, <sup>†</sup> P<0.05, ns - not significant (T test).

-35-

Example 2Inhibition of MHC-I Antigen Presentation by a  
Defect in Ubiquitin Conjugation

The rate-limiting step in the complete degradation  
5 of most cellular proteins is their covalent conjugation  
with ubiquitin (Hershko and Ciechanover, 1992;  
Rechtsteiner, 1987). This step marks the proteins for  
rapid hydrolysis by a 26S (1500 kD) proteolytic complex  
that contains a 20S (700 kD) degradative particle called  
10 the proteasome (Goldberg, 1992; Goldberg and Rock,  
1992). In addition to completely hydrolyzing cell  
proteins, some system in the cytosol generates antigenic  
peptides from endogenously synthesized cellular and  
viral proteins (Townsend et al., 1985; Townsend et al.,  
15 1986; Morrison et al., 1986; Moore et al., 1988; Spies  
and Demars, 1991; Powis et al., 1991). These peptides  
bind to newly synthesized class I major  
histocompatibility complex (MHC) molecules in the  
endoplasmic reticulum and peptide-class I complexes are  
20 then transported to the cell surface for presentation to  
cytotoxic T cells (Yewdell and Bennink, 1992; Monaco,  
1992). Precisely how antigenic peptides are produced is  
unknown, although indirect evidence suggests a role for  
proteolytic particles (LMP) closely resembling and  
25 perhaps identical to the 20S (700 kD) proteasome  
(Goldberg and Rock, 1992; Monaco 1992; Parham, 1990;  
Yang et al., 1992). Applicants therefore tested if  
the ATP-ubiquitin- dependent proteolytic system is  
involved in MHC class I-restricted antigen (Ag)  
30 presentation. Using cells that exhibit a temperature-  
sensitive defect in ubiquitin conjugation, Applicants  
have found that the nonpermissive temperature inhibited

-36-

class I-restricted presentation of ovalbumin (OVA) introduced into the cytosol, but did not affect presentation of an OVA peptide synthesized from a minigene. These results implicate the  
5 ubiquitin-dependent proteolytic pathway in the production of antigenic peptides.

Ts20 cells, a chemically mutagenized variant of the Chinese hamster lung cell line E36, express a thermolabile ubiquitin-conjugating enzyme E1 (Kulka et  
10 al., 1988). E1 catalyzes the ATP-dependent activation of ubiquitin, an essential first step in conjugation of ubiquitin to cellular proteins (Hershko and Ciechanover, 1992; Rechtsteiner, 1987). Ubiquitin-protein conjugation and protein degradation are reduced in ts20  
15 cells at nonpermissive temperature (41°C) (Kulka et al., 1988; Gropper et al., 1991). E1 is irreversibly inactivated in ts20 cells at 41°C.

To determine whether blocking ubiquitin conjugation affected class I-restricted Ag presentation, ts20 and  
20 E36 cells were transfected with the cDNA genes for H-2K<sup>b</sup> and ICAM-1 to enable the cells to present Ag to the OVA-specific, K<sup>b</sup>-restricted murine T-cell hybridoma line RF33.70 (Rock et al., 1990). The transfected cell  
lines, ts20.10.2 (mutant) and E36.12.4 (wild-type), were  
25 incubated at 41°C or 37°C for 1 hour before introducing OVA into the cytosol by osmotic lysis of pinosomes (Townsend et al., 1986). Incubations during and after the introduction of OVA were performed at 37°C; this eliminated temperature as a variable between the groups  
30 during and after their exposure to OVA. The cells were allowed to process the Ag for 1 hour before Ag processing was stopped by aldehyde fixation. Incubation

-37-

at 41°C greatly decreased presentation of OVA by mutant (Figure 5A) but not wild-type (Figure 5B) cells.

Several hypotheses can be suggested to explain these results. These possibilities were examined. The antigen presenting cells (APCs) above acquired OVA by pinocytosis (Townsend et al., 1986). Therefore, presentation of OVA by mutant cells could have been inhibited if exposure to 41°C blocked pinocytosis. Mutant cells incubated at 41°C or 37°C did not differ in their uptake of extracellular fluid ( $0.94 \pm 0.27$  nl/ $10^6$  cells at 41°C versus  $0.96 \pm 0.47$  nl/ $10^6$  cells at 37°C, mean  $\pm$  SD of four experiments) as determined by the uptake of poly(vinylpyrrolidone) (PVP), a commonly used marker of fluid-phase pinocytosis (Wiley and McKinley, 1987). The incubation conditions with PVP were identical to those described above for OVA. Thus, the failure of mutant cells to present OVA was not due to a defect in pinocytosis.

Alternatively, the inability of mutant cells incubated at 41°C to present cytosolic OVA could have been due to a block in the generation or presentation of the antigenic OVA peptide- $K^b$  complexes. Incubation at 41°C did not affect the ability of mutant (Figure 5C) or wild-type (Figure 5D) cells to present exogenous OVA<sub>257-264</sub> peptide (amino acids 257-264 of ovalbumin) added to the medium. Under these conditions, the synthetic OVA<sub>257-264</sub> peptide (which represents the naturally processed epitope from ovalbumin) binds directly to  $K^b$  on the cell surface (Falk et al., 1991; Rock et al., 1992). Therefore, presentation of OVA peptide- $K^b$  complexes at the APC surface was not significantly affected by blocking ubiquitin conjugation.

-38-

After antigenic peptides bind to newly synthesized class I molecules, the peptide-class I complexes are transported through the Golgi complex to the cell surface for presentation to T cells. Therefore,

5 Applicants examined the effects of 41°C on the synthesis and transport of H-2K<sup>b</sup> in mutant cells. Following 1 hour incubation at 41°C or 37°C, cells were radiolabeled with [<sup>35</sup>S]-methionine, and H-2K<sup>b</sup> molecules were sequentially immunoprecipitated with a mAb (Y-3)

10 (Townsend et al., 1990) that is specific for assembled K<sup>b</sup> molecules. This was followed by precipitation with rabbit anti-heavy chain antisera (exon 8) (Smith and Barber, 1990). Similar amounts of assembled H-2K<sup>b</sup> were immunoprecipitated from mutant cells incubated at the

15 two temperatures (Figure 6A). Applicants also found at both temperatures similar amounts of free heavy chains (Figure 6A) and  $\beta_2$ -microglobulin that were potentially available for assembly with peptide.

To analyze the transport of assembled K<sup>b</sup> molecules

20 through the Golgi complex, cells were incubated at 41°C or 37°C for 1 hour and then pulse-radiolabeled and chased at 37°C. H-2K<sup>b</sup> molecules were immunoprecipitated and subjected to two-dimensional IEF/SDS-PAGE. Temperature did not affect the degree of charge or

25 molecular mass heterogeneity of K<sup>b</sup> molecules in either mutant or wild-type cells (Figure 6B). Since changes in charge and molecular mass during the intracellular maturation of class I molecules are associated with complex sugar additions in the Golgi complex, these

30 findings indicate that assembly and transport of H-2K<sup>b</sup> occurred normally in the mutant cells following incubation at 41°C. This suggested that inhibition of



-39-

Ag presentation was due to a selective defect in the production of antigenic OVA peptides.

To examine this point further, mutant cells, treated as described in Figure 5, were incubated with OVA or infected with a recombinant vaccinia virus (Ova<sub>257-264</sub>Vac) containing a minigene encoding the OVA<sub>257-264</sub> peptide preceded by an initiating Met. Presentation of the endogenously-synthesized OVA<sub>257-264</sub> peptide was unaffected by incubation at 41°C (Figure 7A), while, as shown earlier, the presentation of OVA was inhibited (Figure 7B). After one hour of infection, the presentation of OVA<sub>257-264</sub> + Kb by the vaccinia infected cells was considerably less than the presentation by cells that were incubated with OVA (compare the 37°C groups in Figure 7A and 7B). Therefore, it is unlikely that overproduction of the OVA<sub>257-264</sub> peptide could account for the lack of effect of the 41°C incubation on presentation by the vaccinia-infected mutant cells (also see Detailed Description of Figure 7). This finding indicates that the reduction in ubiquitin conjugation caused by incubating mutant cells at 41°C did not affect either the transport of OVA<sub>257-264</sub> peptide from the cytosol into the endoplasmic reticulum, the subsequent assembly of the OVA<sub>257-264</sub> peptide and H-2K<sup>b</sup>, or the transport of the class I-peptide complex to the cell surface.

The simplest interpretation of the present findings is that ubiquitin conjugation plays a critical role in the processing of OVA for class I restricted presentation. In related studies, Applicants found that OVA added to cell-free extracts was degraded by the ubiquitin-dependent pathway, since this process required

-40-

ATP, ubiquitin, a proteasome fraction from rabbit reticulocytes, and free amino groups on the ovalbumin.

The presence of two MHC-encoded proteins in the proteasome has led to the hypothesis that the proteasome is involved in processing cellular and viral Ags (Parham, 1990; Yang et al., 1992). Recent data, however, indicate that these subunits are not essential for class I-restricted Ag presentation (personal communication from Hammerling et al.). Since ubiquitin-conjugated proteins are degraded by the 26S proteasome complex (Goldberg, 1992; Goldberg and Rock, 1992), these findings directly implicate this structure in processing of an Ag for class I-restricted presentation. Thus, the immune system utilizes the cell's two primary and phylogenetically old degradative pathways to supply antigenic peptides: the ATP-ubiquitin-proteasome dependent pathway for class I and the vacuolar pathway for class II-restricted presentation.

## 20 Detailed Description of Figure 5

Figure 5 shows MHC class I-restricted presentation of OVA by mutant (ts20.10.2) and wild-type (E36.12.4) cells. Ts20.10.2 (A and C) and E36.12.4 (B and D) cells were incubated at 41°C (filled) or 37°C (open) and then incubated with (A and B) or without (C and D) OVA. The indicated number of APCs incubated with OVA were cultured with RF33.70 (A and B), or control APCs ( $5 \times 10^4$ ) were cultured with RF33.70 and the indicated concentration of OVA<sub>257-264</sub> peptide (C and D).

30 Ts20.10.2 (mutant) and E36.12.4 (wild-type) cells ( $2 \times 10^6$  cells/ml) were incubated for 1 hour at 41°C or 37°C. For introducing OVA into the cytosol, the cells

-41-

were incubated for 10 minutes at 37°C with 20 mg/ml OVA in hypertonic media, treated with hypotonic media, and then washed with ice-cold, serum-free RPMI 1640, as previously described (Townsend et al., 1986; Michalek et al., 1991). The washed APCs ( $2 \times 10^6$  cells/ml) were incubated for 1 hour at 37°C to allow for the expression of processed Ag on the cell surface. During this 37°C incubation, some recovery of ubiquitin conjugating activity ( $16 \pm 10\%$  mean  $\pm$  SD of three experiments) did occur in the mutant cells that had been previously incubated at 41°C. APCs were fixed with 1% paraformaldehyde to prevent further recovery of ubiquitin conjugation and/or Ag processing and added to duplicate microcultures (200  $\mu$ l) with RF33.70 ( $10^5$  cells), as previously described (Michalek et al., 1991). Control APCs were as described above except OVA was omitted from the hypertonic media. RF33.70 is stimulated to produce IL-2 upon recognition of processed OVA + K<sup>b</sup> on the surface of APCs (Rock et al., 1990b). The microcultures were prepared, incubated, and assayed for IL-2 content as previously described (Michalek et al., 1991).

The H-2K<sup>b</sup> cDNA, pBG367-K<sup>b</sup>, was kindly provided by Dr. Gerald Waneck. The vector pcDL-SR $\alpha$ 296 was provided by Dr. Naoko Arai (Takebe et al., 1988). The H-2K<sup>b</sup> cDNA was subcloned into the XhoI/BamHI sites of the pcDL-SR $\alpha$ 296 vector. Murine ICAM-I cDNA in the pH<sub>g</sub>APr-1-neo vector was provided by Drs. Hedrick and Brian (Siu et al., 1989). E36 and ts20 cells were cotransfected with H-2K<sup>b</sup> and ICAM-I using the Lipofectin reagent (BRL) and selection with G418 (GIBCO) were performed as previously described (Dang et al., 1990), except ts20 cells were grown at 37°C following transfection. G418-resistant

-42-

clones were screened for expression of ICAM-I and H2K<sup>b</sup> with mAb YN1/1.7.4 (Dang et al., 1990) and B8-24-3 (Kohler et al., 1981), respectively. The transfected cell lines ts20.10.2 and E36.12.4 were passaged at 31°C and 37°C, respectively.

#### Detailed Description of Figure 6

Figure 6 shows the effect of temperature on the synthesis and maturation of H-2K<sup>b</sup>. In Figure 6A, mutant (ts20.10.2) and wild-type (E36.12.4) cells were incubated for 1 hour at 41°C or 37°C and then metabolically radiolabeled for 15 minutes at 37°C. H-2K<sup>b</sup> was sequentially immunoprecipitated with Y-3 (Jones and Janeway, 1981) followed by precipitation with anti-exon 8 (Smith and Barber, 1990) (a kind gift from Dr. Brian Barber, University of Toronto) and analyzed by one-dimensional SDS-PAGE. Y-3 recognizes a conformational epitope formed by the  $\alpha 1$  and  $\alpha 2$  domains of H-2K<sup>b</sup> and binds only assembled H-2K<sup>b</sup> molecules, whereas the anti-exon 8 antiserum was raised against the C-terminal cytoplasmic domain of H-2K<sup>b</sup> (encoded by exon 8) and binds both assembled H2K<sup>b</sup> molecules and free heavy chains. The positions of H-2K<sup>b</sup> heavy chains (H) and  $\beta_2$ -microglobulin (L) and of the relative molecular mass standards ( $M_r \times 10^{-3}$ ) are indicated in Figure 6.

Forty million cells were incubated for 1 hour at 41°C or 37°C in methionine-free media and then radiolabeled for 15 minutes at 37°C with 0.7 mCi of [<sup>35</sup>S]-methionine (>1000 Ci/mmol, NEN). Detergent lysates, immunoprecipitations and SDS-PAGE were performed essentially as described (Townsend et al., 1990), except that additional preclearing steps with

-43-

IgG-sorb (5 times) and PA-sepharose (1 time) were included before and after the first immunoprecipitation, respectively.

In Figure 6B, ts20.10.2 and E36.12.4 cells were  
5 incubated for 1 hour at 41°C or 37°C, metabolically pulse-radiolabeled for 10 minutes at 37°C, and then chased for 50 minutes at 37°C. H-2K<sup>b</sup> was immunoprecipitated with Y-3 and analyzed by two-dimensional IEF/SDS-PAGE. Immunoprecipitates made  
10 at the 0 time point of the chase were analyzed and showed a single immature heavy chain spot, which chased into the more acidic and larger molecular mass forms shown above. The heavy chains of H-2K<sup>b</sup> are indicated by the H, and the  $\beta_2$ -microglobulin is indicated by the L.  
15 Cells were incubated and pulsed-radiolabeled for 10 minutes with [<sup>35</sup>S]-methionine as described above. The cells were then chased in the presence of cold methionine (15 mg/ml) for 50 minutes at 37°C. Cell lysates were prepared and immunoprecipitations with Y-3  
20 were performed as described above. For two-dimensional IEF/SDS-PAGE, the immunoprecipitates were analyzed by using the Immobiline DryStrip Kit (Pharmacia). Gels were incubated in Autofluor (Nalional Diagnostics), and radiolabeled proteins were visualized by autoradiography  
25 at -80°C.

#### Detailed Description of Figure 7

Figure 7 shows MHC class I-restricted presentation of endogenously-synthesized OVA<sub>257-264</sub> peptide. Ts20.10.2 cells were incubated at 41°C (filled) or 37°C  
30 (open) for 1 hour and then either infected with Ova<sub>257-264</sub>Vac for 1 hour at 37°C (Figure 7A) or incubated

-44-

with OVA (Figure 7B). The indicated number of APCs were incubated with RF33.70.

Ts20.10.2 cells ( $7 \times 10^5$  cells/ml) were incubated for 1 hour at 41°C or 37°C and then infected with  
5 recombinant vaccinia virus (10 PFU/cell) for 1 hour at 37°C (Figure 7A). Ts20.10.2 cells were incubated at 41°C and 37°C, treated with OVA, and then incubated at 37°C as described above (see Detailed Description of Figure 5). The APCs infected with vaccinia virus or  
10 incubated with OVA were fixed with 1% paraformaldehyde and incubated in microcultures with RF33.70 ( $10^5$  cells) as described in the Detailed Description of Figure 5. The microcultures were prepared, incubated, and assayed for IL-2 content as also described therein. APCs  
15 infected with a recombinant vaccinia virus containing an influenza nucleoprotein gene did not stimulate RF33.70.

Time-course experiments with Ova<sub>257-264</sub>Vac showed that the amount of OVA<sub>257-264</sub> peptide-K<sup>b</sup> complexes on the surface of cells infected for 1 hour was limiting  
20 because cells infected for 3 hours were 64-fold more efficient at stimulating RF33.70.

Ova<sub>257-264</sub>Vac was constructed by inserting a synthetic oligonucleotide behind the vaccinia virus p7.5 early/late promoter in pSc11 (Chakrabati et al., 1985),  
25 which was modified such that the restriction sites SalI and NotI were substituted for the SmaI site. The oligonucleotide:

5'-TCGACCACCATGCTCTATAATAAACTTTGAGAAGTTATAGTGACCATGGGC-3'

3'-GGTGGTACAGATATTATTTGAAACTCTCAATATCACTGGTACCCGCCGG-5',

30 (Seq. ID #1), consisted of a SalI site, Kozak's consensus sequence for efficient translation, an initiation codon, nucleotides encoding the peptide S I I N F E K L (Seq. ID #2), two stop codons, and a NotI

-45-

site, which is not present in pSC11 and therefore provides a simple method for determining the presence of the insert in the plasmid. After strand annealing, the synthetic oligonucleotide was phosphorylated with T4 polynucleotide kinase. pSC11 was digested with SalI and NotI, purified, and ligated with the oligonucleotide using T4 ligase. Homologous recombination of the plasmid with vaccinia virus, selection, and propagation of recombinants was then performed as described (Moss and Earl, 1991).

### Example 3

#### Inhibition of MHC-1 Presentation By Chymostatin

##### **ANTIGEN PRESENTING CELLS**

The antigen-presenting cells (APCs) in this experiment were a mouse B lymphoblastoid cell line (LB27.4) that was passaged in media supplemented with normal mouse serum (1%) and lipopolysaccharide (10 µg/ml X 72 hours). Except for the inclusion of the lipopolysaccharide, this is described in Rock et al., 1990b.

##### **INHIBITOR TREATMENT AND ANTIGEN LOADING**

The LB27.4 cells were washed serum free and incubated in the presence or absence of chymostatin (200 µg/ml; Boehringer Mannheim, Indianapolis, IN) for one hour at 37°C. The cells were then resuspended in Electroporation buffer (phosphate buffered saline, 1 mM Hepes, 0.4 M mannitol; chymostatin was also added to the appropriate groups) containing either the antigen ovalbumin (30 mg/ml) or a synthetic peptide corresponding to amino acids 257-264 of ovalbumin. The resuspended cells were then subjected to electroporation

-46-

on the CELL-PORATOR from Gibco BRL (Gaithersburg, MD) at 4°C with setting of high ohms, capacitance 1180. The cells were washed 4 times at 4°C and incubated at 37°C for two hours in the continued presence or absence of  
5 chymostatin. After this two hour incubation, the cells were fixed with 1% paraformaldehyde for 10 minutes at room temperature, followed by washing.

#### ANTIGEN PRESENTATION ASSAY

The indicated number of fixed antigen-presenting  
10 cells were incubated with  $10^5$  RF33.70 cells (an OVA + K<sup>b</sup> specific T-T hybridoma) in duplicate 200  $\mu$ l microcultures. After 18 hours at 37°C, an aliquot (100  $\mu$ l) of culture supernatant was harvested and assayed for IL-2 content using HT-2 cells, as described in Rock et  
15 al., 1990a and 1990b.

#### Detailed Description of Figure 8

Figure 8 shows MHC-I presentation of ovalbumin (OVA left panel) and an ovalbumin peptide (right panel). In the left panel, APCs were treated with (open circles) or  
20 without (closed circles) chymostatin and electroporated with ovalbumin. In the right panel. APCs were treated with (open circles) or without (closed circles) chymostatin and electroporated with ovalbumin peptide.

The left panel demonstrates that chymostatin  
25 inhibits the presentation of ovalbumin with class I MHC molecules. The right panel demonstrates that chymostatin does not inhibit the presentation of electroporated peptide. This result indicates that the inhibition of antigen presentation is occurring through  
30 chymostatin inhibition of the processing of the ovalbumin protein into the ovalbumin peptide.



-47-

EXAMPLE 4Isolation of an Endogenous Inhibitor of the Proteasome

As described in Example 7, a 40 kDa polypeptide regulator of the proteasome, which inhibits the  
5 proteasome's proteolytic activities, has been purified from reticulocytes and shown to be an ATP-binding protein whose release appears to activate proteolysis. The isolated inhibitor exists as a 250 kDa multimer and is quite labile (at 42°C). It can be stabilized by the  
10 addition of ATP or a non-hydrolyzable ATP analog, although the purified inhibitor does not require ATP to inhibit proteasome function and lacks ATPase activity. The inhibitor has been shown to correspond to an essential component of the 1500 kDa proteolytic complex.  
15 If reticulocytes are depleted of ATP, the 1500 kDa UC DEN is not found. Instead, Ganoth et al. identified three components, designated CF-1, CF-2 and CF-3, (*J. Biol. Chem.* 263:12412-12419 (1988)). The inhibitor isolated as described herein appears identical to CF-2 by many  
20 criteria. These findings indicate the idea that the inhibitor plays a role in the ATP-dependent mechanism of the UC DEN complex. It is possible, for example, that during protein breakdown, within the 1500 kDa complex, ATP hydrolysis leads to functional release of the 40 kDa  
25 inhibitor, temporarily allowing proteasome activity, and that ubiquitinated proteins trigger this mechanism.

The purified factor has been shown to inhibit hydrolysis by the proteasome of both a fluorogenic tetrapeptide and protein substrates. When the  
30 inhibitor, the proteasome and partially purified CF-1 were mixed in the presence of ATP and  $Mg^{2+}$ , the 1500 kDa

-48-

complex was reconstituted and degradation of Ub-<sup>125</sup>I-lysozyme occurred.

Isolation of this inhibitor of the multiple peptidase activities of the proteasome makes available  
5 an attractive site for pharmacological intervention. As described subsequently, this provides a natural inhibitor whose structural and functional features can be assessed to provide information useful in developing proteasome inhibitors.

#### 10 Materials and Methods

DEAE-cellulose (DE-52), CM-cellulose (CM-52), and phosphocellulose (P11) were obtained from Whatman. Ub-conjugating enzymes (E1, E2 and E3) were isolated using  
15 Ub-sepharose affinity column chromatography (Hershko et al., *J. Biol. Chem.* 258:8206-8215 (1983)), and were used to prepare Ub-<sup>125</sup>I-lysozyme conjugates (Hershko and Heller, *Biochem. Biophys. Res. Comm.* 128:1079-1086 (1985)). All other materials used were as described in the previous examples.

#### 20 Purification

Rabbit reticulocytes induced by phenylhydrazine injection were prepared (as described previously or purchased from Green Hectares (Oregon, WI). They were depleted of ATP by incubation with 2, 4-dinitrophenol  
25 and 2-deoxyglucose as described (Ciechanover et al., *Biochem. Biophys. Res. Comm.* 81:1100-1105 (1978)). Lysates were then prepared and subjected to DE-52 chromatography. The protein eluted with 0.5M KCl (Hershko et al., *J. Biol. Chem.*, 258:8206-8214 (1983))  
30 was concentrated using ammonium sulfate to 80% saturation, centrifugated at 10,000 x g for 20 minutes,

-49-

and suspended in 20 mM Tris-HCl (pH 7.6), 1 mM DTT (Buffer A). Following extensive dialysis against the same buffer, the protein (fraction II) was either stored at -80°C in 0.5 mM ATP or fractionated further.

5        Fraction II (-200 mg) was applied to a Ub-sepharose column, and the Ub-conjugating enzymes were specifically eluted (Hershko et al., *J. Biol. Chem.*, 258:8206-8214 (1983)) and used in making Ub-lysozyme (Hershko and Heller, *Biochem. Biophys. Res. Comm.* 128:1079-1086 (1985)).

10        The unabsorbed fraction was brought to 38% saturation using ammonium sulfate and mixed for 20 minutes, as described by Ganoth et al. (Ganoth et al., *J. Biol. Chem.* 263:12412-12419 (1988)). The precipitated proteins were collected by centrifugation

15        at 10,000 x g for 15 minutes. The pellet was resuspended in Buffer A and brought again to 38% saturation with ammonium sulfate. The precipitated material was collected as above and then suspended in Buffer A containing 10% glycerol. After dialysis

20        against this buffer, the 0-38% pellet was chromatographed on a Mono-Q anion exchange column equilibrated with Buffer A containing 10% glycerol. The protein was eluted using a 60 ml linear NaCl gradient from 20 to 400 mM. Fractions which inhibited the

25        peptidase activity of the proteasome were pooled, concentrated, and then chromatographed on a Superose 6 (HR 10/30) gel filtration column equilibrated in Buffer A containing 100 mM NaCl and 0.2 mM ATP. The column was run at a flow rate of 0.2 ml/minute, and 1 ml fractions

30        were collected. Further purification of the inhibitor was achieved by a second more narrow Mono-Q chromatographic gradient (from 50 to 300 mM NaCl), which yielded a sharp peak of inhibitor where only the 40 kDa

-50-

band was visible after SDS-PAGE and Coomassie staining. Fractions with inhibitory activity against the proteasome were pooled and dialyzed against Buffer B which contained 20 mM  $\text{KH}_2\text{PO}_4$  (pH 6.5), 10% glycerol, 1 mM DTT and 1 mM ATP. The sample was then applied to a 2 ml phosphocellulose column equilibrated in Buffer B. The column was washed with 4 ml of this buffer, followed by 4 ml of this buffer, followed by 4 ml of Buffer B containing either 20, 50, 100, 400 or 600 mM NaCl.

10 To obtain partially pure CF-1, the Mono-Q fractions that eluted from 100 to 240 mM NaCl were pooled, concentrated to 1 ml and applied to a superose 6 column equilibrated in Buffer A containing 100 mM NaCl and 0.2 mM ATP. The fractions eluting at approximately 600 kDa

15 were used as the CF-1 containing fraction.

The proteasome was isolated from the supernatants of the two 38% ammonium sulfate precipitations. The supernatants were brought to 80% saturation with ammonium sulfate and mixed for 20 minutes. The precipitated protein was collected by centrifugation, resuspended in Buffer A, and dialyzed extensively against this buffer. The proteasome was isolated by Mono-Q anion exchange chromatography followed by gel filtration on superose 6 as described previously

20 (Driscoll and Goldberg, *Proc. Natl. Acad. Sci., USA* 86:789-791 (1989)).

25

The 1,500 kDa proteolytic complex was generated by incubating reticulocyte fraction II at 37°C for 30 minutes in the presence of 2 mM ATP, 5 mM  $\text{MgCl}_2$  in 50 mM Tris-HCl (pH 7.6). After precipitation with ammonium sulfate to 38% saturation, the pellet was collected at 10,000 x g for 10 minutes, suspended in Buffer A, and

30

-51-

isolated by Mono-Q anion exchange and superose 6 chromatography.

### Assays

Inhibition of the proteasome was measured by  
5 preincubating individual column fractions with the  
proteasome in the presence of 1 mM ATP at 37°C for 10  
minutes. After preincubation, the reaction tubes were  
placed on ice, and either  $^{125}\text{I}$ -lysozyme or Suc-LLVY-MCA  
was added. Reactions were carried out at 37°C for 60  
10 minutes with  $^{125}\text{I}$ -lysozyme or 10 minutes with Suc-LLVY-  
MCA. Protein hydrolysis was assayed by measuring  
production of radioactivity soluble in 10%  
trichloroacetic acid, and peptide hydrolysis by the  
release of methylcoumaryl-7-amide (Driscoll and  
15 Goldberg, *Proc. Natl. Acad. Sci., USA* 86:789-791  
(1989)). Degradation of Ub-conjugated  $^{125}\text{I}$ -lysozyme  
was assayed at 27°C for 60 minutes. Reactions contained  
either 5 mM EDTA or 2 mM ATP and 5 mM  $\text{MgCl}_2$  and were  
terminated by adding 10% trichloroacetic acid.

20

### RESULTS

#### Isolation of the Inhibitor

To understand how the proteasome is regulated in  
vivo and how it functions in the Ub-conjugate-degrading  
complex, Applicants attempted to isolate factors which  
25 influence its activity. Reticulocyte fraction II was  
separated using ammonium sulfate into fractions which  
precipitated with either 0-38% or 40-80%. The latter  
fraction was used to isolated proteasomes. The  
particles (obtained in this way from ATP-depleted  
30 reticulocytes) showed appreciable activity against  $^{125}\text{I}$ -

-52-

lysozyme and Suc-LLVY-MCA which was independent of ATP (Eytan et al., *Proc. Natl. Acad. Sci., USA* 86:7751-7755 (1989); Driscoll and Goldberg, *J. Biol. Chem.* 265:4789-4792 (1990)). Neither the proteasome nor the 0-38% fraction showed significant activity against Ub-conjugated  $^{125}\text{I}$ -lysozyme (Eytan et al., *Proc. Natl. Acad. Sci., USA* 86:7751-7755 (1989); Driscoll and Goldberg, *J. Biol. Chem.* 265:4789-4792 (1990)). However, as reported previously, ATP-dependent degradation of the ubiquitinated lysozyme was observed after the protease and the 0-38% fraction were preincubated together in the presence of ATP.

The 0-38% precipitated material was then separated using Mono-Q anion exchange and each fraction assayed for its ability to influence the proteasome activity against Suc-LLVY-MCA or  $^{125}\text{I}$ -lysozyme. Column fractions were preincubated with the proteasome for 10 minutes and then either substrate was added. None of the column fractions by itself showed significant hydrolytic activity. A peak of inhibitory activity was eluted around 240 to 280 mM NaCl. It significantly decreased proteolytic activity against both substrates. Hydrolysis of lysozyme and the peptide was inhibited to a similar extent.

To purify the inhibitory activity further, the active fractions were pooled and chromatographed by gel filtration. The inhibitor eluted as a sharp peak with an apparent molecular weight of about 100-150 kDa.

The active fractions were then pooled and assayed for their ability to inhibit the substrate hydrolyzing activities of the proteasomes. With increasing inhibitor concentration, proteasome activity decreased in a linear manner with both  $^{125}\text{I}$ -lysozyme and Suc-LLVY-

-53-

MCA as substrates, although the degree of the inhibition was highly variable between preparations.

The Inhibitor Is A Component of the 1,500kDa Proteolytic Complex

- 5        Like the inhibitor, one component of the 1,500 kDa proteolytic complex (CF-2) has been reported to have a molecular weight of about 250 kDa. To test if the inhibitor corresponds to CF-2, the inhibitor obtained by gel filtration was subjected to phosphocellulose
- 10 chromatography. Eytan et al. had noted that CF-2 has little affinity for phosphocellulose and elutes with less than 100 mM NaCl. Accordingly, Applicants found that the inhibitory activity was recovered in the flow through and 20 mM NaCl eluate (i.e., in the region where
- 15 CF-2 activity was reported). Individual phosphocellulose fractions were then assayed for their ability to reconstitute degradation of ubiquitinated lysozyme. Individually or combined, the proteasome and CF-1 containing fraction did not support rapid breakdown
- 20 of ubiquitinated lysozyme. However, when this mixture was combined with the peak of the inhibitor activity, the rate of Ub-<sup>125</sup>I-lysozyme degradation increased sharply. No other phosphocellulose fractions stimulated this process.
- 25        These results suggest strongly that the inhibitor corresponds to CF-2 and thus is essential for hydrolysis of Ub-ligated proteins. One unusual property of CF-2 is that it is quite labile upon heating to 42°C, but is stabilized by ATP (Ganoth et al., *J. Biol. Chem.*
- 30 263:12412-12419 (1988)). To test further if the inhibitor of the proteasome corresponds to CF-2, the purified inhibitor was preincubated at 42°C with or

-54-

without ATP or the nonhydrolyzable analog, AMPPNP. The proteasome was added and after 10 minutes, peptidase activity was assayed. The degree of inhibition decreased rapidly during preincubation without  
5 nucleotide added. The presence of either ATP or AMPPNP prevented this loss of activity. Furthermore, the ability of this material to reconstitute degradation of Ub-conjugated lysozyme also decreased rapidly during incubation of 42°C, and the addition of ATP or AMPPNP  
10 (not shown) prevented this activation. Since the inhibition and reconstitution of Ub-conjugate degradation showed similar inactivation kinetics and were stabilized similarly by ATP, these two functions probably reside in a single molecule which appears to  
15 bind ATP.

Although ATP stabilizes the inhibitory factor, it is not essential for inhibition of the proteasome. After preincubation of the inhibitor with proteasome for up to 20 minutes with or without ATP, a similar degree  
20 of inhibition was observed. Nevertheless, because of the stabilization by ATP, this nucleotide was routinely added to all incubations.

When analyzed by SDS-PAGE, the inhibitor preparations showed a major band of 40 kDa. To test  
25 whether this 40 kDa subunit corresponded to any subunit of the 1,500 kDa complex, the 1,500 kDa complex was formed by incubation of fraction II with  $Mg^{2+}$ -ATP and isolated by anion exchange and gel filtration chromatography. SDS-PAGE of these active fractions  
30 indicated many polypeptides similar to those previously reported for this complex (Hough et al., *J. Biol. Chem.* 262:8303-8313 (1987); Ganoth et al., *J. Biol. Chem.* 263:12412-125419 (1988); Eytan, E. et al., *Proc. Natl.*



-55-

*Acad. Sci. USA* 86:7751-7755 (1989)). However, a readily apparent band of 40 kDa was evident in this fraction. To further address the question of proteins associated with the proteasome, fraction II was immunoprecipitated using an anti-proteasome monoclonal antibody and analyzed by SDS-PAGE. Ub-conjugate degrading activity had previously been shown to be removed upon immunoprecipitation of fraction II (Matthews et al., *Proc. Natl. Acad. Sci. USA* 86:2597-2601 (1989)). Upon SDS-PAGE of the immunoprecipitates, Applicants observed the characteristic set of proteasome subunits ranging from 20 to 34 kDa, along with other higher molecule weight bands. Importantly, a 40 kDa band, similar to that of the inhibitor and similar to that seen in the partially purified complex was detected in the immunoprecipitate.

-56-

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-57-

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-63-

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

- 64 -  
SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: THE PRESIDENT AND FELLOWS OF HARVARD COLLEGE AND  
DANA FARBER CANCER INSTITUTE
- (ii) TITLE OF INVENTION: Role of ATP-Ubiquitin-Dependent  
Proteolysis in MHC-1 Restricted Antigen Presentation And  
Inhibitors Thereof
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Sterne, Kessler, Goldstein & Fox
  - (B) STREET: 1100 New York Avenue, Suite 600
  - (C) CITY: Washington
  - (D) STATE: D.C.
  - (E) COUNTRY: USA
  - (F) ZIP: 20005-3934
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: TO BE ASSIGNED
  - (B) FILING DATE: 27-JAN-1994
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Goldstein, Jorge A.
  - (B) REGISTRATION NUMBER: 29,021
  - (C) REFERENCE/DOCKET NUMBER: 1448.003PC00
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (202) 371-2600
  - (B) TELEFAX: (202) 371-2540

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 50 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: both

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCGACCACCA TGTCTATAAT AAACCTTTGAG AAGTTATAGT GACCATGGGC

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  - (B) TYPE: amino acid
  - (D) TOPOLOGY: both

- 65 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ser Ile Ile Asn Phe Glu Lys Leu  
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: both

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1..4
- (D) OTHER INFORMATION: /note= "The sequence Leu Val Val Tyr is  
part of a modified peptide having the structure  
Suc-Leu Val Val Tyr-MCA."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu Leu Val Tyr  
1

- 66 -

CLAIMS

The invention claimed is:

1. A method for inhibiting MHC-I antigen presentation in an antigen-presenting cell, comprising  
5 contacting the cell with an inhibitor that inhibits proteolysis of intracellular proteins by the ATP-ubiquitin-dependent pathway, under conditions suitable for the inhibitor to enter the cell.
2. The method of Claim 1, wherein the inhibitor  
10 inhibits a peptidase of the proteasomes, said peptidase having an activity which is selected from the group consisting of:
  - a) cleavage after basic residues;
  - b) cleavage after hydrophobic residues; and
  - 15 c) a combination of a) and b).
3. The method of Claim 2, wherein the inhibitor is a peptide aldehyde.
4. The method of Claim 3, wherein the peptide aldehyde  
20 is selected from the group consisting of chymostatin and leupeptin.
5. The method of Claim 1, wherein the inhibitor inhibits ubiquitin conjugation of the intracellular proteins.

- 67 -

6. The method of Claim 5, wherein the inhibitor inhibits a protein selected from the group consisting of:
- a) E1, also referred to as ubiquitin activating enzyme;
  - b) E2, also referred to as ubiquitin-carrier protein; and
  - c) E3, also referred to as ubiquitin-protein ligase.
7. The method of Claim 6, wherein the inhibitor of E1 is a substrate analog of ubiquitin adenylate.
8. A method for inhibiting cytolytic immune responses in a mammalian tissue, comprising contacting the tissue with an inhibitor that inhibits proteolysis of intracellular proteins by the ATP-ubiquitin dependent pathway, under conditions suitable for the inhibitor to enter antigen-presenting cells in the tissue.
9. The method of Claim 8, wherein the inhibitor inhibits a step in the proteolysis selected from the group consisting of:
- a) cleavage by a peptidase of the proteasomes, which is selected from the group consisting of cleavage after basic residues and cleavage after hydrophobic residues;
  - b) ubiquitin conjugation; and
  - c) a combination of a) and b).

10. The method of Claim 9, wherein the inhibitor inhibits a protein selected from the group consisting of:
- 5 a) E1, also referred to as ubiquitin activating enzyme;
  - b) E2, also referred to as ubiquitin-carrier protein; and
  - c) E3, also referred to as ubiquitin-protein ligase.
- 10 11. A method for inhibiting cytolytic immune responses in an individual, comprising administering to the individual an inhibitor that inhibits proteolysis of intracellular proteins by the ATP-ubiquitin dependent pathway, under conditions suitable for the inhibitor to enter antigen-presenting cells in the individual.
- 15 12. The method of Claim 11, wherein the inhibitor inhibits a step in the proteolysis selected from the group consisting of:
- 20 a) cleavage by a peptidase of the proteasomes, which is selected from the group consisting of cleavage after basic residues and cleavage after hydrophobic residues;
  - 25 b) ubiquitin conjugation; and
  - c) a combination of a) and b).

13. A method of therapy or prevention of an autoimmune disease in an individual, comprising administering to the individual an inhibitor that inhibits proteolysis of intracellular proteins by the ATP-ubiquitin-dependent pathway, under conditions suitable for the inhibitor to enter antigen-presenting cells in the individual.
14. The method of Claim 13, wherein the inhibitor inhibits a step in the proteolysis selected from the group consisting of:
- a) cleavage by a peptidase of the proteasomes, which is selected from the group consisting of cleavage after basic residues and cleavage after hydrophobic residues;
  - b) ubiquitin conjugation; and
  - c) a combination of a) and b).
15. A method for reducing rejection of foreign tissue by an individual, comprising administering to the individual an inhibitor that inhibits proteolysis of intracellular proteins by the ATP-ubiquitin-dependent pathway, under conditions suitable for the inhibitor to enter antigen-presenting cells in the individual.

- 70 -

16. The method of Claim 15, wherein the inhibitor inhibits a step in the proteolysis selected from the group consisting of:
- a) cleavage by a peptidase of the proteasomes, which is selected from the group consisting of cleavage after basic residues and cleavage after hydrophobic residues;
  - b) ubiquitin conjugation; and
  - c) a combination of a) and b).
- 10 17. The method of Claim 15, wherein the foreign tissue is a transplanted organ or graft.



- 1 / 15

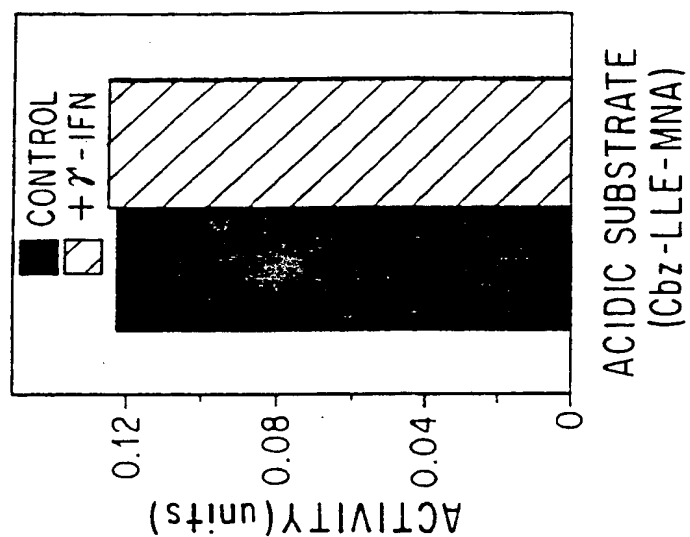


FIG. 1c

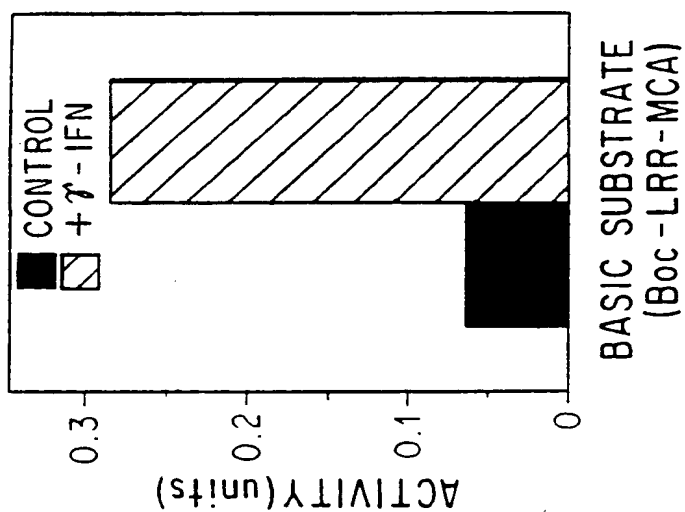


FIG. 1b

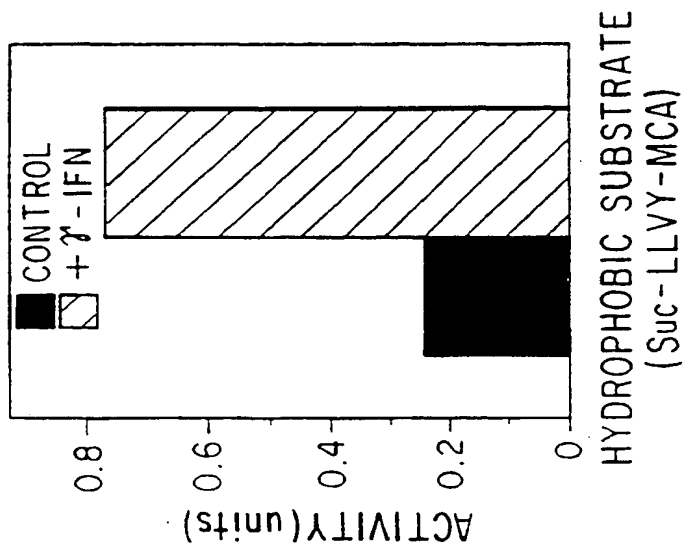


FIG. 1a

- 2 / 15

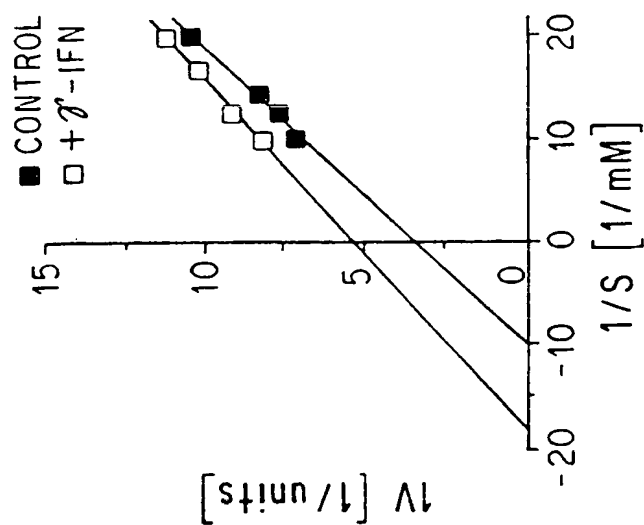


FIG. 1f

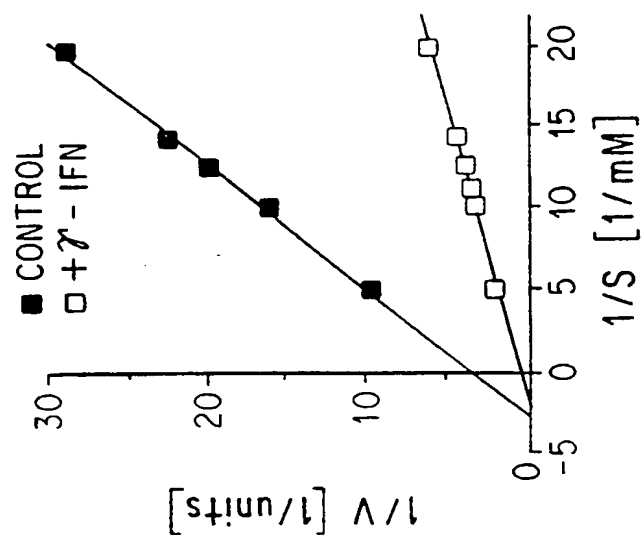


FIG. 1e

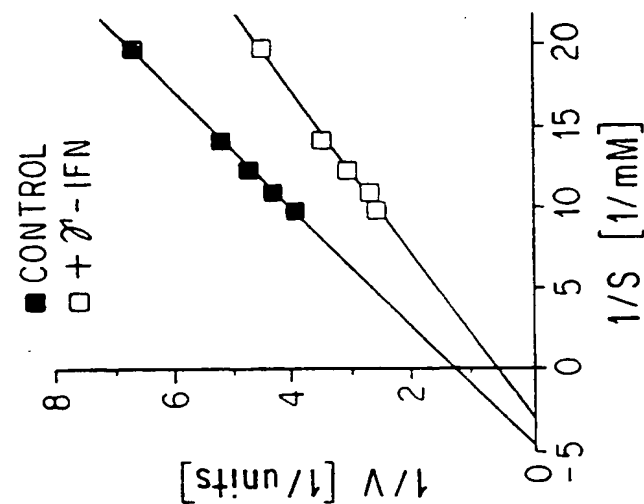


FIG. 1d

3/15

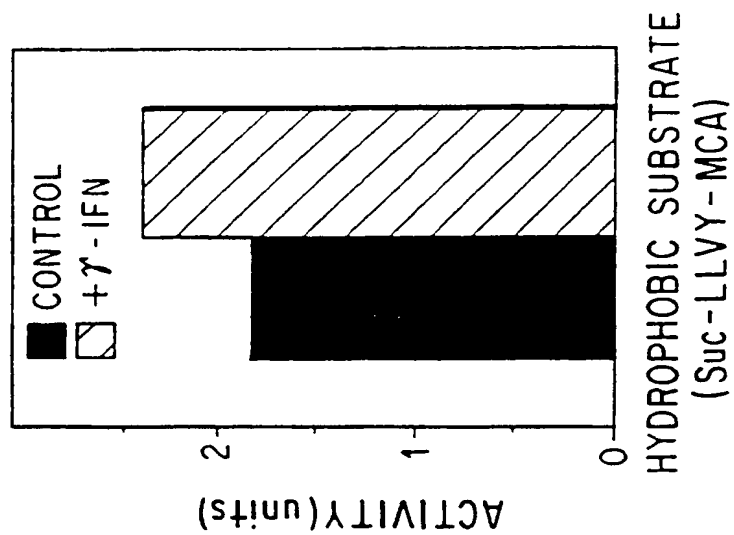


FIG. 2b

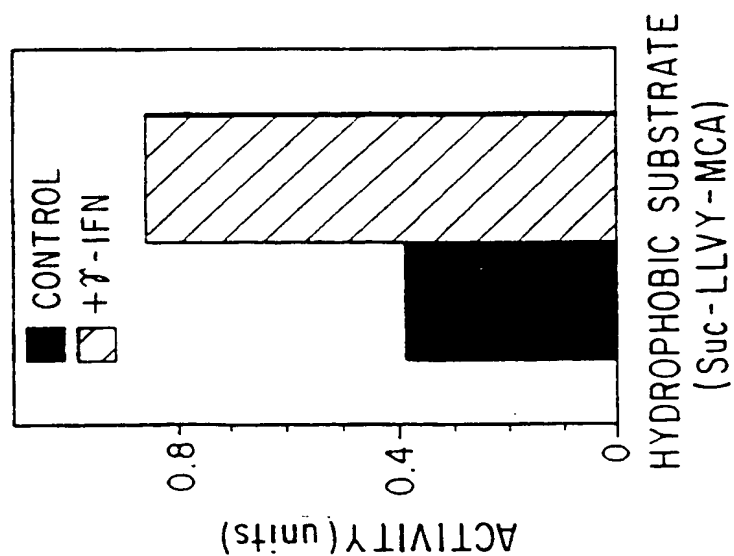


FIG. 2a

4/15

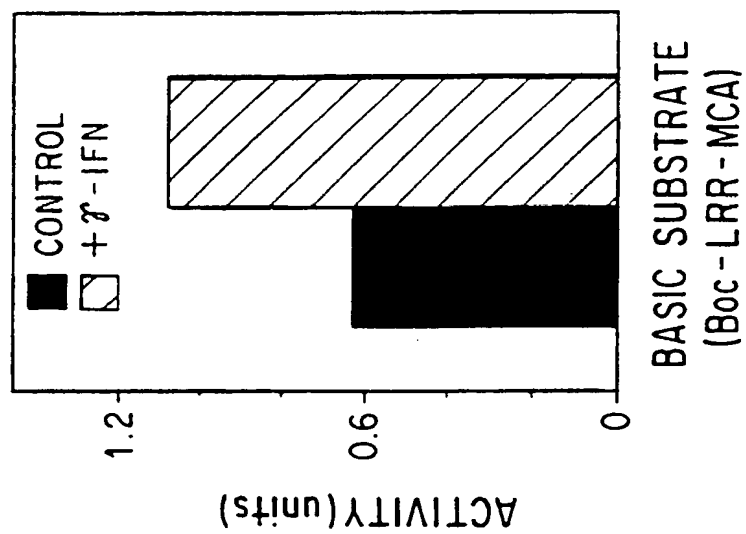


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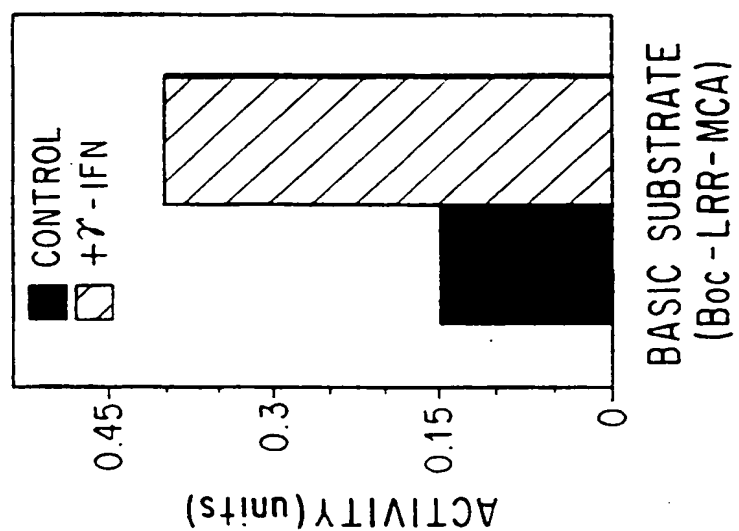


FIG. 2c

5/15

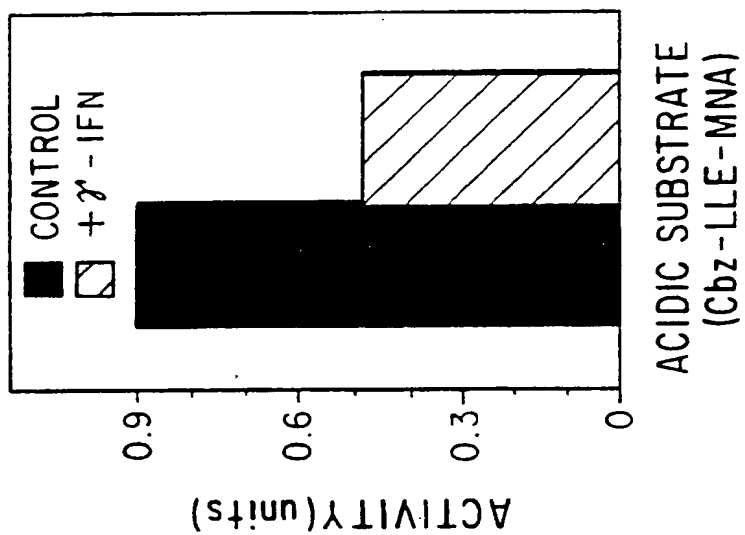


FIG. 2f

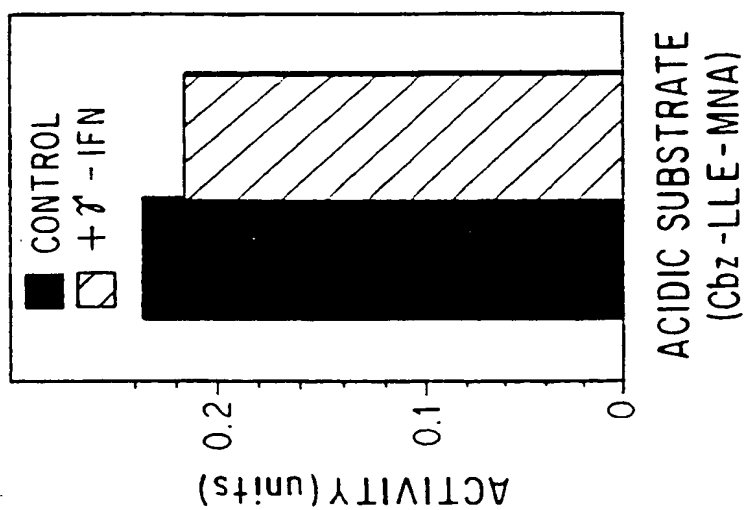


FIG. 2e

6 / 15

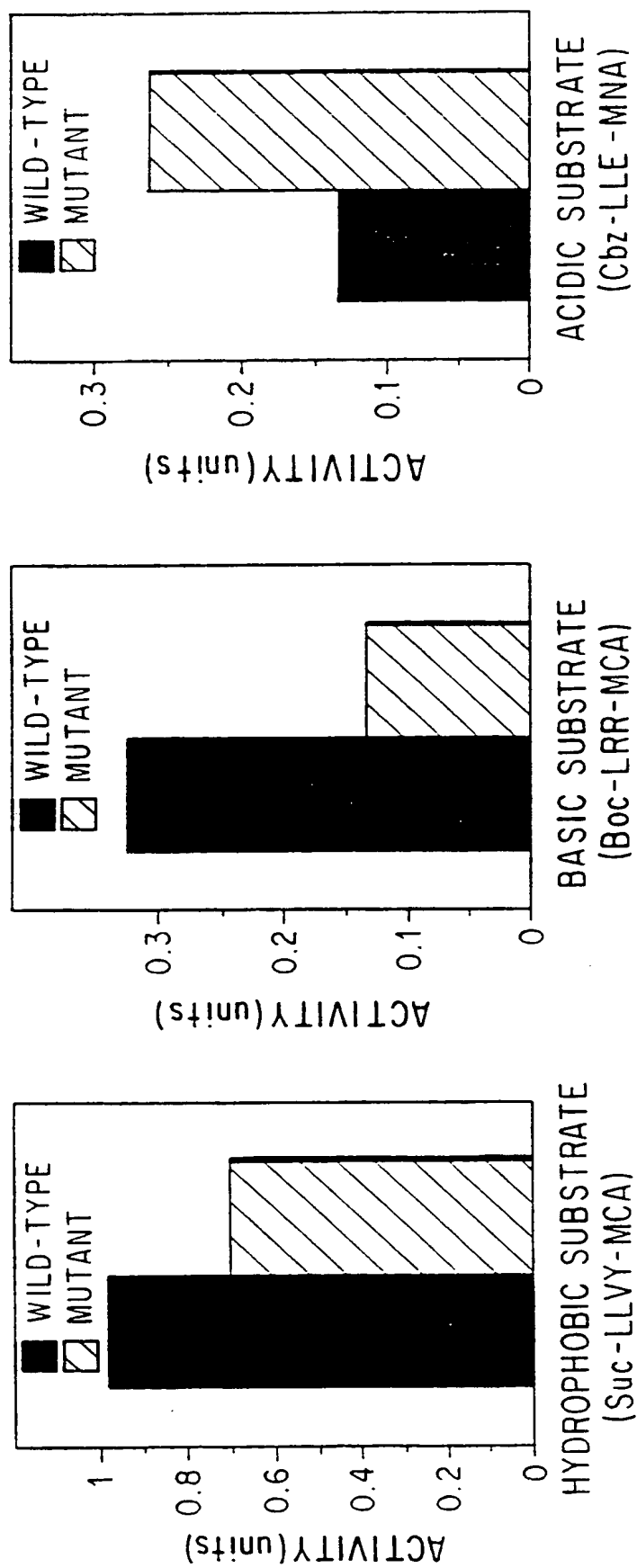


FIG. 3a

FIG. 3b

FIG. 3c

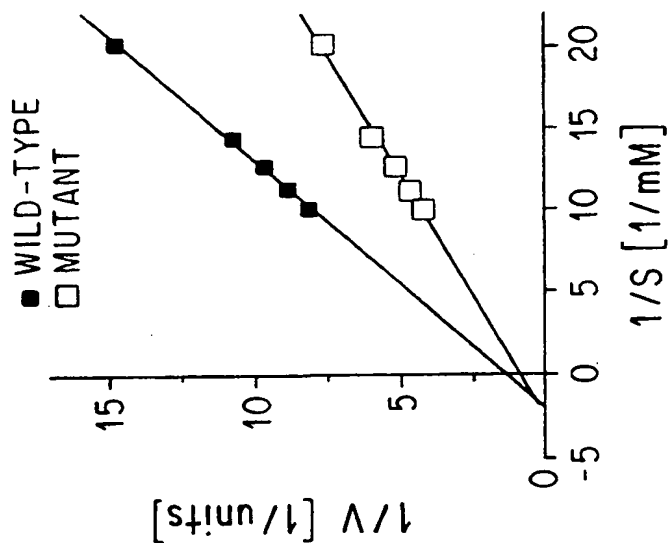


FIG. 3f

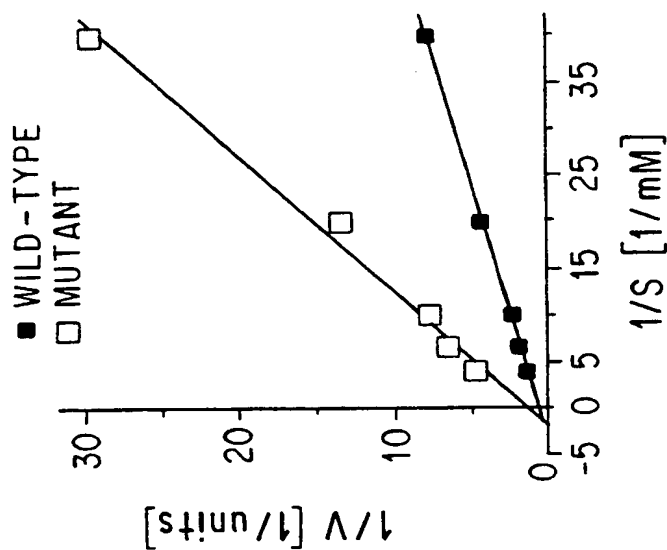


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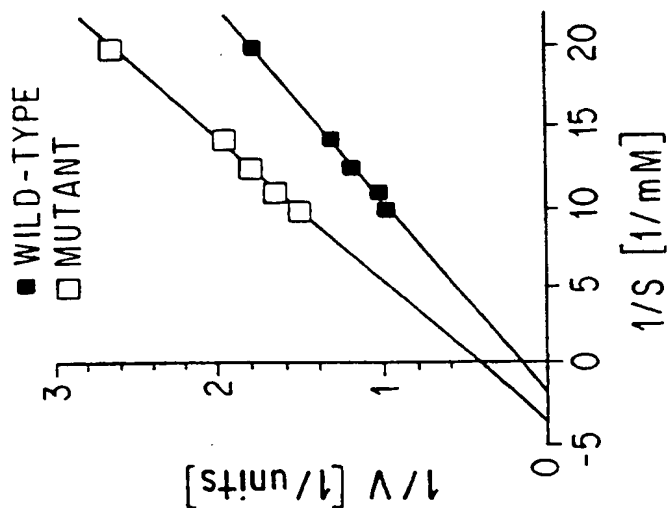


FIG. 3d

- 8 / 15

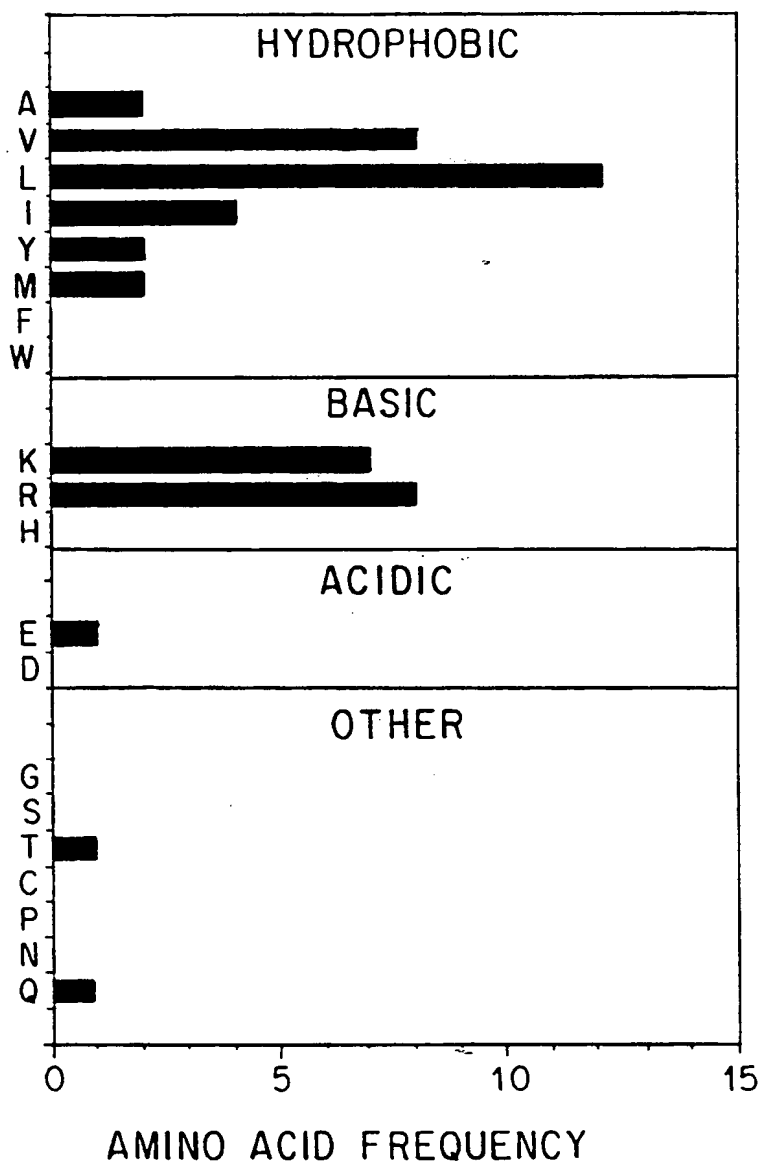


FIG. 4a



- 9 / 15

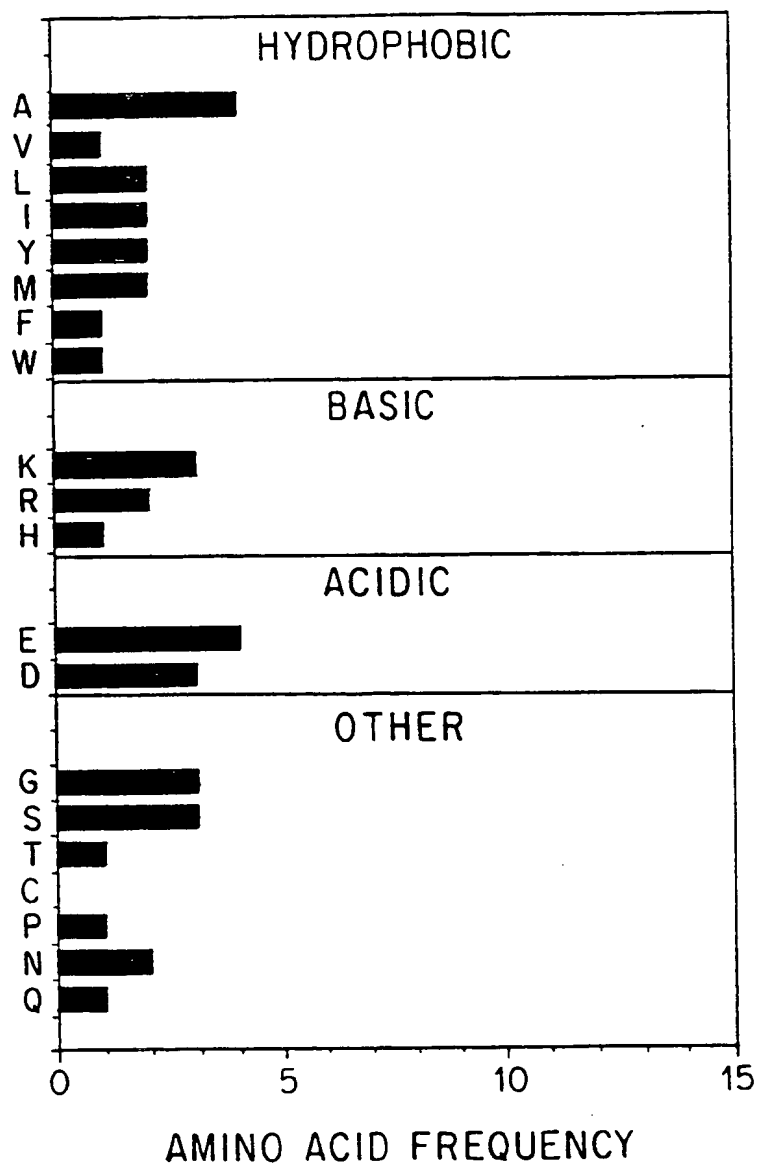


FIG. 4b

10 / 15

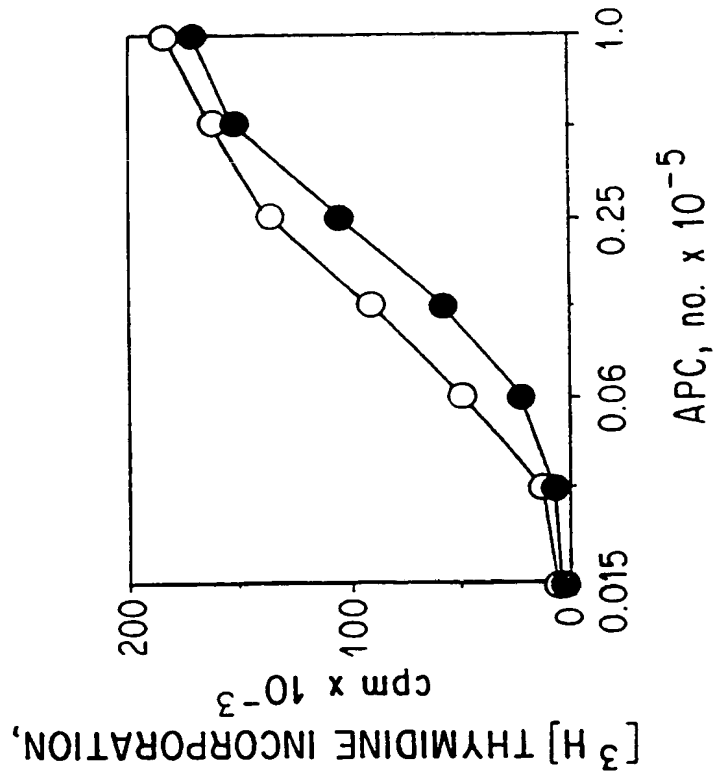


FIG. 5b

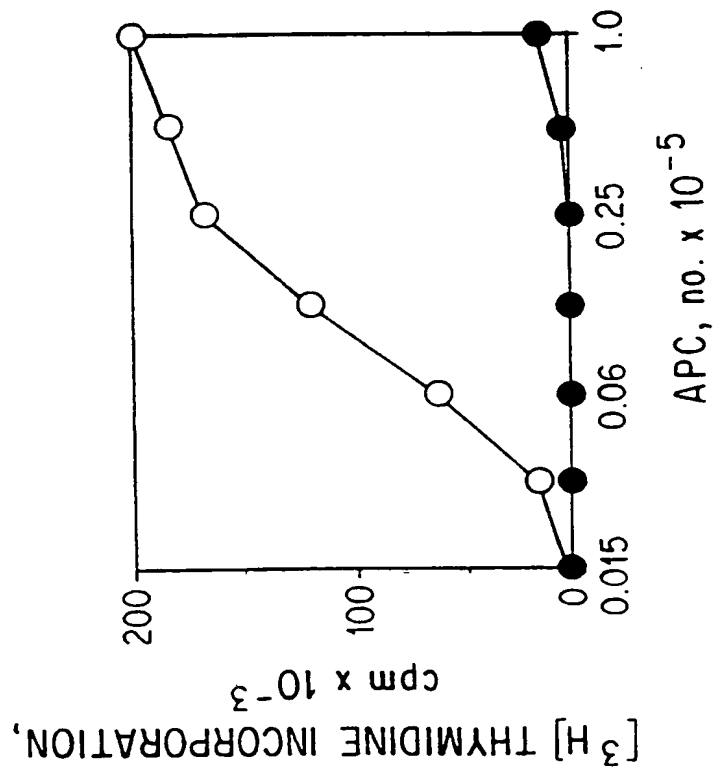
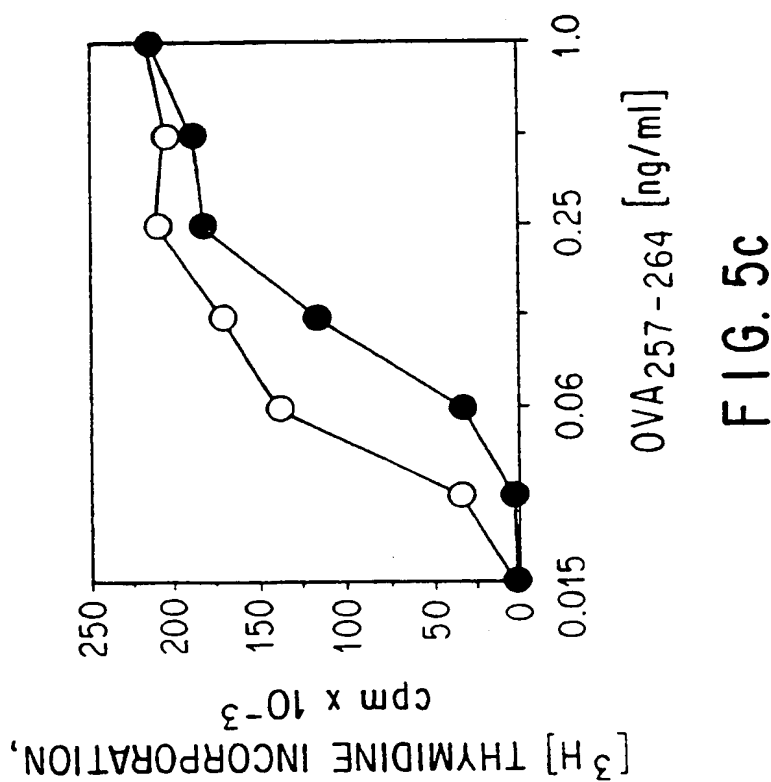
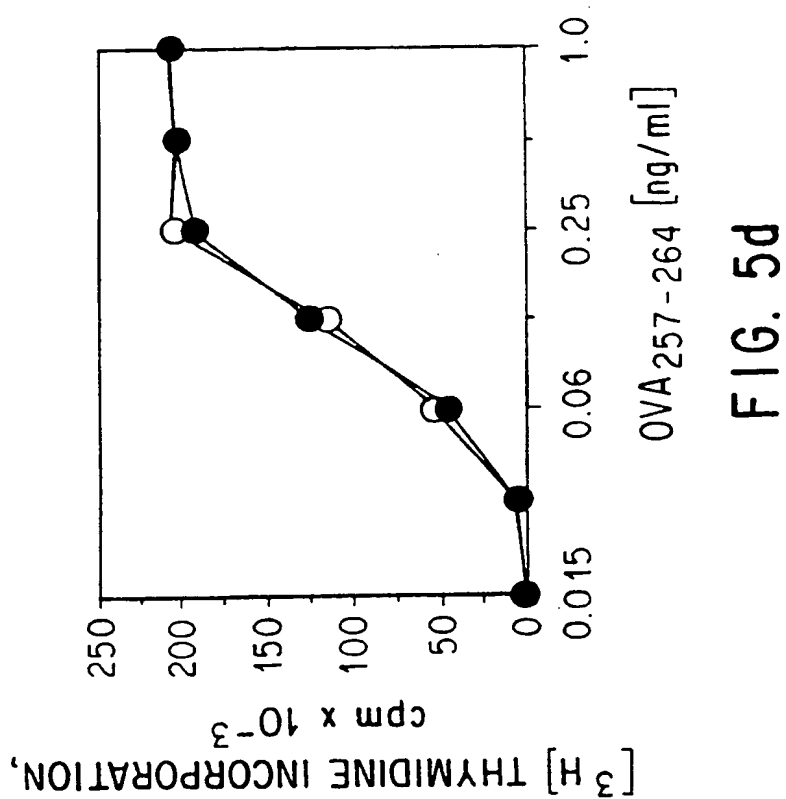


FIG. 5a

11 / 15



12/15

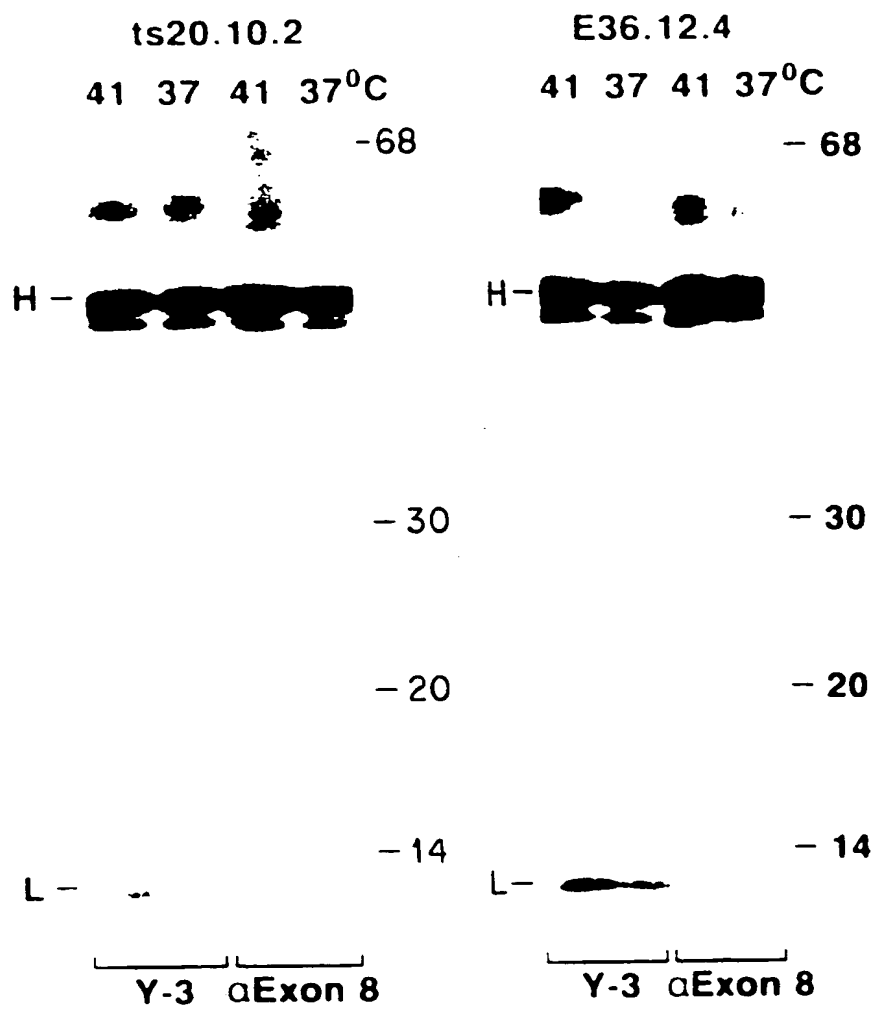


FIG. 6a

FIG. 6b

13/15

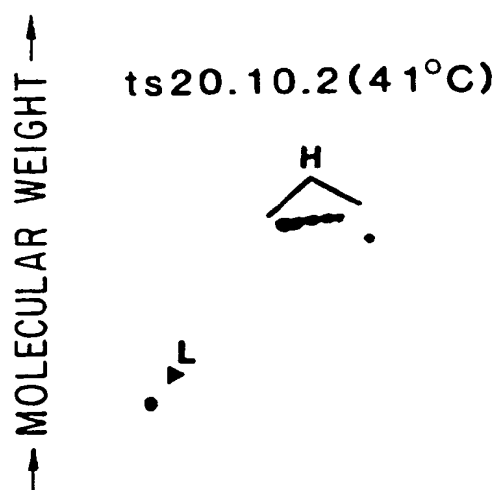


FIG. 6c

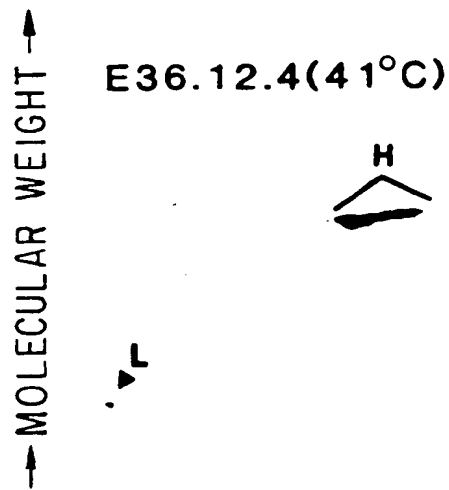


FIG. 6d

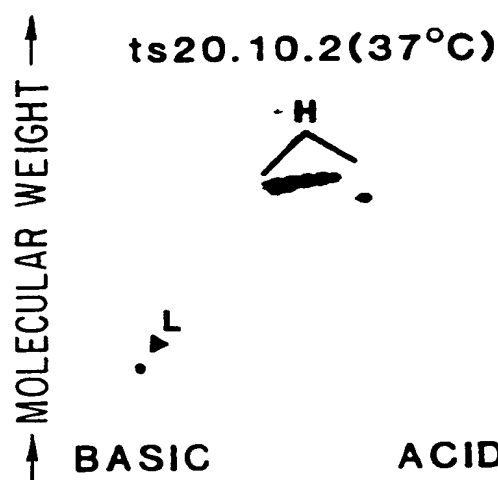


FIG. 6e

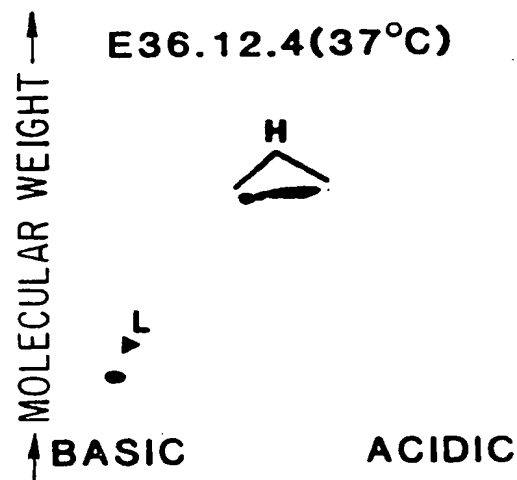


FIG. 6f

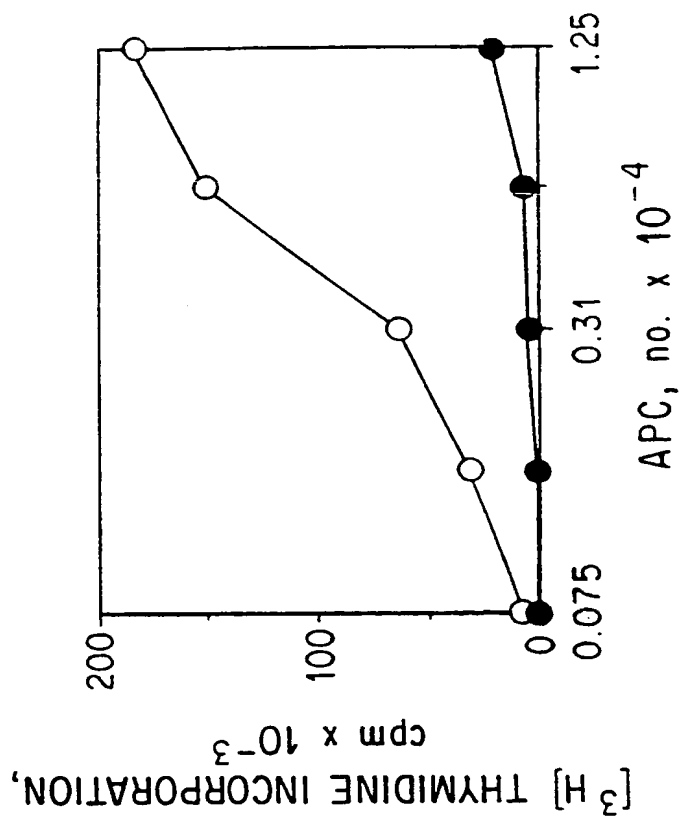


FIG. 7b

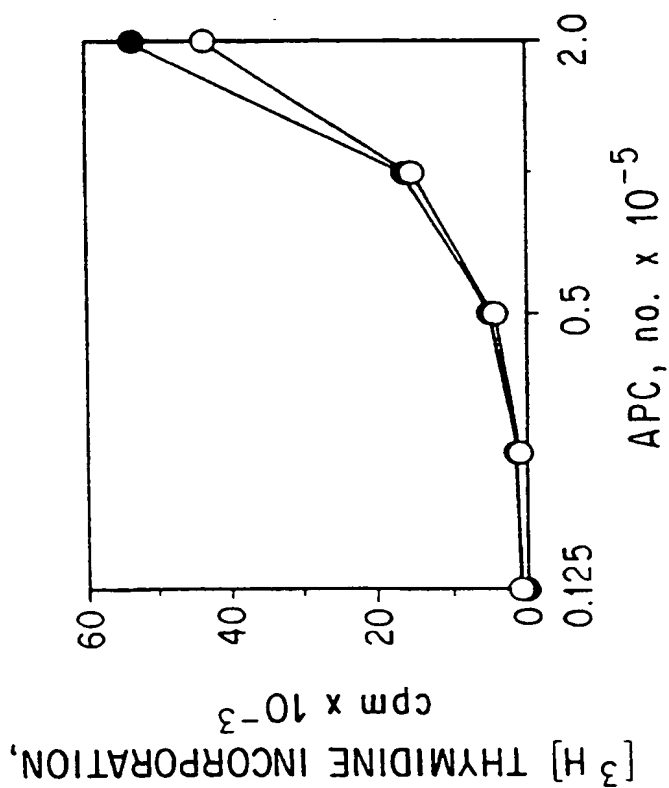


FIG. 7a

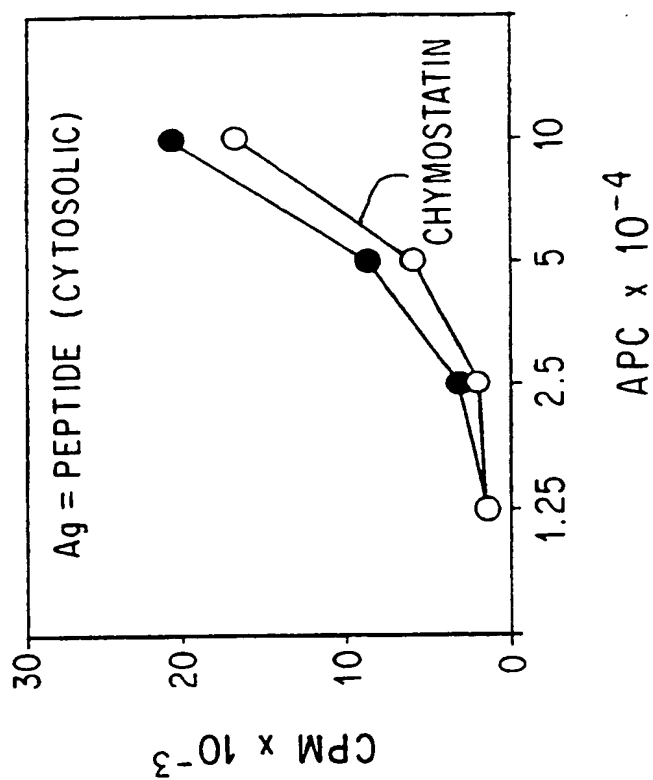


FIG. 8b

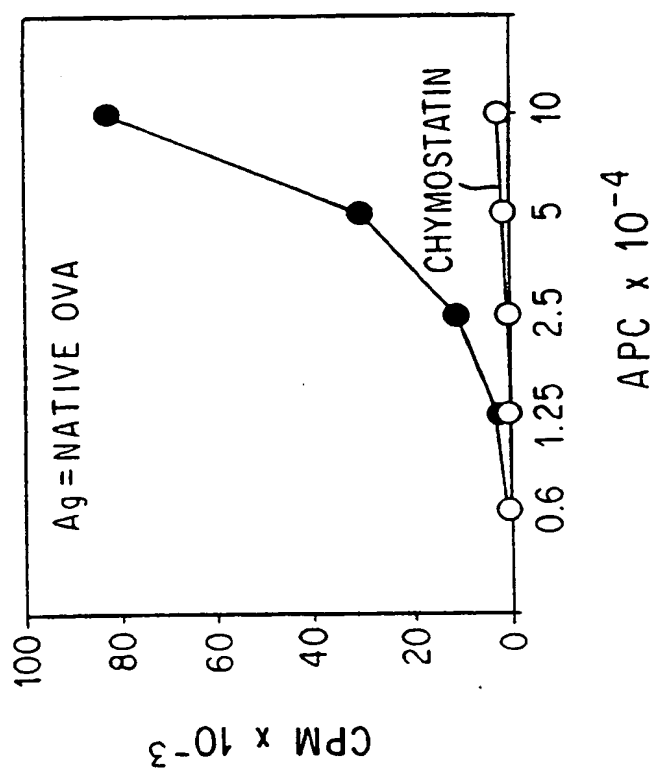


FIG. 8a

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/01183

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 37/00, 37/48, 37/64, 39/00

US CL : 424/88, 94.1; 514/2, 8

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88, 94.1; 514/2, 8; 530/826

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	U.S. A, 4,390,629 (GOLDBERG ET AL.) 28 June 1983. See entire document.	1-17
A	W.E. Paul, "Fundamental Immunology", Third Edition, published 1993 by Ravan Press, Ltd. (NY), pages 653-663. See entire document.	1-17
A	Ann. Rev. Cell Biol., Vol. 3, issued 1987, M. Rechsteiner, "Ubiquitin-mediated pathways for intracellular proteolysis". Pages 1-30. See entire document.	1-17
Y	Proc. Natl. Acad. Sci. (USA), Vol. 89, issued September 1992, L.D. Falo, et al., "Serum proteases alter the antigenicity of peptides presented by class I major histocompatibility complex molecules." Pages 8347-8350. See abstract.	1-17



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A document defining the general state of the art which is not considered to be part of particular relevance	*X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E earlier document published on or after the international filing date	*Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z document member of the same patent family
*O document referring to an oral disclosure, use, exhibition or other means	
*P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

16 MARCH 1994

Date of mailing of the international search report

19 APR 1994

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

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## INTERNATIONAL SEARCH REPORT

International application No.  
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## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochem. Soc. Trans., Vol. 19 No. 3, issued 1991, A. Hershko, "The ubiquitin pathway of protein degradation and proteolysis of ubiquitin-protein conjugates". Pages 726-9. See abstract.	1-17
Y	Science, Vol. 249, issued 24 August 1990, K. L. Rock, et al., "Presentation of exogenous antigen with class I major histocompatibility complex molecules. Pages 918-921. See abstract.	1-17
Y	Am. J. Respir. Cell Mol. Biol., Vol. 7, issued 1992, A.L. Schwartz, et al., "Ubiquitin-mediated protein modification and degradation. Pages 463-468. See abstract.	1-17
Y	Experientia, Vol. 48, issued 1992, T. Sommer, et al., "Genetic analysis of ubiquitin-dependent protein degradation". Pages 172-178. See abstract.	1-17

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